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BIOLOGICAL BULLETIN

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INSTRUCTIONS TO AUTHORS

THE BIOLOGICAL BULLETIN accepts original research reports of intermediate length on a variety of subjects of biological interest. In general, these papers are either of particular interest to workers at the Marine Biological Laboratory, or of outstanding general significance to a large number of biologists throughout the world. Normally, review papers (except for a limited number of solicited review papers which may be accepted after formal refereeing), very short papers (less than five printed pages), preliminary notes, and papers which describe only a new technique or method without presenting substantial quantities of data resulting from the use of the new method cannot be accepted for publication. A paper will usually appear within four months of the date of its acceptance.

The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts which do not conform will be returned to authors for correction before review.

1. ***Manuscripts.*** Manuscripts, including figures, should be submitted in triplicate. (Xerox copies of photographs are not acceptable for review purposes.) The original manuscript must be typed in double spacing (*including* figure legends, footnotes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. Manuscripts should be proofread carefully and errors corrected legibly in black ink. Pages should be numbered consecutively. Margins on all sides should be at least 1 inch (2.5 cm). Manuscripts should conform to the *Council of Biology Editors Style Manual*, 4th Edition (Council of Biology Editors, 1978) and to American spelling. Unusual abbreviations should be kept to a minimum and should be spelled out on first reference as well as defined in a footnote on the title page. Manuscripts should be divided into the following components: Title page, Abstract (of no more than 200 words), Introduction, Materials and Methods, Results, Discussion, Acknowledgments, Literature Cited, Tables, and Figure Legends. In addition, authors should supply a list of words and phrases under which the article should be indexed.

2. ***Figures.*** Figures should be no larger than 8½ by 11 inches. The dimensions of the printed page, 5 by 7⅜ inches, should be kept in mind in preparing figures for publication. We recommend that figures be about 1½ times the linear dimensions of the final printing desired, and that the ratio of the largest to the smallest letter or number and of the thickest to the thinnest line not exceed 1:1.5. Explanatory matter generally should be included in legends, although axes should always be identified on the illustration itself. Figures should be prepared for reproduction as either line cuts or halftones. Figures to be reproduced as line cuts should be unmounted glossy photographic reproductions or drawn in black ink on white paper, good-quality tracing cloth or plastic, or blue-lined coordinate paper. Those to be reproduced as halftones should be mounted on board, with both designating numbers or letters and scale bars affixed directly to the figures. All figures should be numbered in consecutive order, with no distinction between text and plate figures. The author's name and an arrow indicating orientation should appear on the reverse side of all figures.

3. ***Tables, footnotes, figure legends, etc.*** Authors should follow the style in a recent issue of *The Biological Bulletin* in preparing table headings, figure legends, and the like. Because of the high cost of setting tabular material in type, authors are asked to limit such material as much as possible. Tables,

Continued on Cover Three

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A COMPARATIVE STUDY OF THE NET METABOLIC BENEFITS DERIVED FROM THE UPTAKE AND RELEASE OF FREE AMINO ACIDS BY MARINE INVERTEBRATES[1]

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ABSTRACT

A comparative study was undertaken to evaluate the overall significance of free amino acid (FAA) uptake and release in the biology of 21 species of marine invertebrates, representing 7 phyla. Measurements of transport fluxes were made in defined media using the fluorescamine method, corrected for ammonia excretion, and related to measured metabolic rates and natural environmental levels. Except for arthropods, in which net uptake was negligible, all the animals exhibited influx of FAA at rates dependent on media levels, and low, constant efflux. Equilibrium concentrations were always found to be less than the mean minimum bay water concentrations. Thus, all must derive benefit to commonly support at least 3–10% of their metabolism—approximately sufficient to provide for the needs of epidermal and associated tissues, including ciliary pumping over gills, tube feet activity, *etc.* Certain forms, including a sand dollar and brittlestar, appear to have become particularly adapted to make use of this resource, which may provide them more sustenance than ingested food. Yet other species support lesser, but still substantial, proportions of their metabolic requirements through these mechanisms. It is concluded that net uptake of FAA is an essentially universal property of soft-bodied marine invertebrates, and it plays an important part in their well-being, especially by providing for much of the energy needs of superficial structures and aiding in metabolite retention.

INTRODUCTION

Many estuarine and marine animals can obtain sustenance not only from the food they eat, but also through the direct assimilation of dissolved organic compounds found in their environments. This fact was originally demonstrated for a large group of diverse soft-bodied species by Stephens and Schinske (1961), although the original supposition was advanced much earlier, by Pütter (1909, 1911). Pütter, however, overstated his case and was largely discredited by the critical review of Krogh (1931) (*cf.* Jørgensen, 1976).

The actual significance of Stephens and Schinske's (1961) observations has also been brought into legitimate question. The concentrations of glycine they observed as being removed from solution were far in excess of normal sea water levels; the participation of micro-organisms could not be ruled out; the release of other amino acids from the animals to perhaps balance glycine uptake was not adequately investigated; and, there was no way of knowing where on the animals the uptake had really taken place. In short, it was not clear whether the observations they

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Abbreviations: FAA—free amino acids.

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reported were a relatively insignificant artifact of the experimental conditions or an indication of an important physiological or ecological phenomenon.

In the last twenty years dozens of reports by a number of different investigators, employing much more elegant techniques, have succeeded in clarifying many of these uncertainties. Uptake of free amino acids (FAA), sugars, and fatty acids from natural concentrations can definitely occur, and its demonstration is, in most cases, little biased by micro-organisms. It is also apparent that similar compounds may be released back into sea water, but usually in comparatively small quantities, and that the uptake proceeds mainly into the exposed epidermal surfaces and not the gut. This body of work has been reviewed by Stephens (1968, 1972), Jørgensen (1976), and Stewart (1979); more recently summarized by Southward *et al.* (1979), Jørgensen (1980), Ferguson (1980a, b), and Stewart (1981); and evaluated in an American Society of Zoologists symposium (Seattle, 1980).

Clearly, the observed uptake of dissolved organics by estuarine and marine invertebrates is not an experimental artifact, but a real physiological process, which must provide advantages to these animals. It is still not clear, however, just what those advantages might be. Most marine animals eat, and by eating would seem to satisfy the bulk of their nutritional needs. Ferguson (1967, 1970), after autoradiographic studies on starfish, suggested that the benefit may not be so much to the whole animal as specifically to the epidermis: periodic eating would serve the nutritional needs of the internal parts, while continuous epidermal uptake would largely provide for the needs of this isolated tissue. A later study, (Ferguson, 1980a) confirmed in one species (*Echinaster*) that the amount of exogenous uptake normally occurring was about adequate to support the epidermal demands, but other experiments (Ferguson, 1980b) failed to show that the epidermis was limited to this source of nutrition: animals were found to be able to survive prolonged periods in near nutrient-free water.

What role, then, is served by exogenous uptake of dissolved organic compounds? This certainly is one of the major physiological and ecological questions remaining in the understanding of the biology of estuarine and marine organisms. The uptake may be a significant component of the total nutrition of some species, especially filter-feeders or smaller individuals. It may indeed be a vital source of sustenance for exposed soft tissues. It may be important in metabolite retention. It may be important in cell volume regulation or in other, as yet unknown, physiological activities. Or, it may be a vestigial relic from a more primitive state.

The full answer to this question may only come gradually, but clearly a first step towards the decipherment of the problem might be found in a modern comparative study which, by using newer, more sophisticated techniques, would go far beyond the basic findings of Stephens and Schinske (1961) in delineating the relative metabolic dependence of diverse species on this source of nutrition. The present account describes such data obtained from 21 species (of seven phyla) all examined within a relatively short period of essentially uniform environmental conditions.

MATERIALS AND METHODS

The study was made feasible by the development and refinement of several procedures, which permitted the rapid accumulation of data with much greater sensitivity, accuracy, and facility than has previously been possible.

Sea water system

Accurate measurements of transport fluxes can only be made from a medium which is much purer than natural sea water, and if the animals and vessels used are well flushed with this medium before the initiation of measurements. Freshly formulated artificial media have proven inadequate for this purpose because their quality is difficult to control and large volumes are needed. Therefore, a recirculating system was developed, which has proven invaluable for fulfilling this need. The system employs biological filtration on aged, granular, activated charcoal, multiple levels of mechanical filtration, oxidative reduction with ozone and UV light, and high levels of aeration. It reduces measurable organics to about 1% of the level of natural sea water. While it has been described briefly previously (Ferguson, 1980a, b), it is pictured more fully in Figure 1. The actual configuration does not appear to be critical except that efficient filtration (Millepore AP20 depth filters or equivalent) must occur both before and after ozonation, and the level of ozone must be adequate, but less than 0.05 mg/l in the final product water. Ozone was supplied from a generator of the design of Honn *et al.* (1976). The major mechanisms of organic removal are believed to be by micro-organisms on the biological filters, adsorption to chemical precipitates in the ozonation chambers, and oxidation. The original water is formulated from commercial synthetic sea salts mixed separately in batches, and added periodically to replace amounts withdrawn for various purposes. The temperature and salinity of the water are carefully adjusted to match that of the environment from which the animals are taken. The system can reduce fluorescamine reactants (measured as glycine) to less than 2×10^{-8} *M*. Healthy animals have been maintained in the water for over six months.

The adjustable syringe pump (SP) included in the system allowed the introduction of defined levels of amino acids to the water before it flowed into experimental vessels containing test animals. The amino acid mixture used for this purpose consisted of 509 mg "Eagle" (tissue culture) minimal amino acids (Difco), 80 mg "Eagle" non-essential amino acids (Difco), and 5 g glycine per liter of stock solution.

Chemical analyses

Amino acid levels were measured by an improved version of the fluorescamine method originally developed by Undenfriend *et al.* (1972). Borate buffer (pH 9.2; 0.5 *M*) was purified by filtration through a 0.45μ membrane filter, and 0.5 ml portions placed in a series of paired, carefully cleaned fluorometer tubes. The first pair of tubes then received 2.5 ml distilled water as a blank, and the second and third pairs similar amounts of 1×10^{-7} and 1×10^{-6} *M* freshly prepared glycine standards. As physical adsorption onto glass can be a problem at lower concentrations of standards, a minimal quantity of the detergent, Brij-35, was added to them, and a consistent pipetting procedure utilizing repeated rinsings was employed. With these precautions, linearity of fluorescence can be obtained.

Experimental samples were withdrawn after several pipette flushings with a 10 ml oral pipette, and 2.5 ml portions added to each of a pair of the prepared tubes (the remainder in the pipette was returned immediately to the experimental vessel). Within a few minutes of collecting the first samples, the blanks, standards, and samples each received 0.5 ml fluorescamine reagent (20 mg/100 ml acetone) injected vigorously with a mechanical pipette and immediately vortexed. After about ten minutes all the tubes were read against the distilled water blanks on a Turner 110 fluorometer equipped with a temperature stabilized door, "7-60" primary and "3" secondary filters, and usually a 3 or 10 × slit and a 10% neutral density filter. Care was maintained to prepare and read subsequent samples of each run with the same time sequence. Concentrations of unknown samples were calculated from the linear slope provided by the two sets of glycine standards.

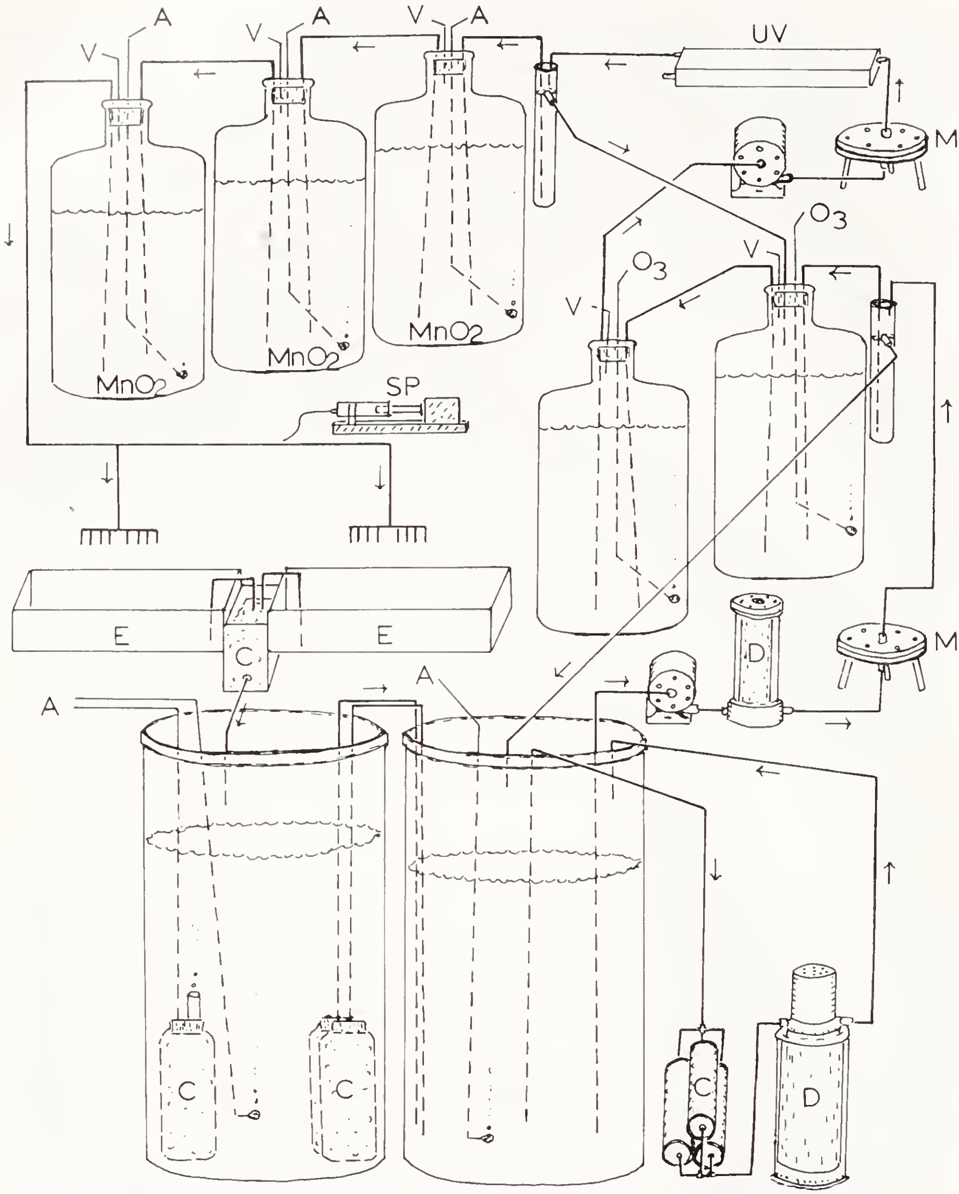

FIGURE 1. Recirculating system for removing organics from sea water media. A, air bubbler, C, granular charcoal filter; D, diatomaceous earth filter; E, experimental platform; M, Millipore depth filter; MnO_2, granular manganese dioxide on bottom of vessel to help breakdown ozone; O_3, ozone bubbler; SP syringe pump for re-introducing FAA; UV, ultraviolet irradiator; V, vent (to sewer drain).

Since the experimental samples were all taken from highly purified water, no preliminary cleaning treatment was necessary for them. Comparative samples of natural bay water, however, were filtered in the field using 0.45μ syringe mounted filters, or filtered (to the same degree) in bulk after returning them to the laboratory.

The fluorescamine procedure is largely specific for primary amines, but ammonia is also known to give a minor response, which probably varies with the details of the analytical procedure used. Jørgensen (1980) has reported a relatively elaborate formula to correct for it. In the present case, ammonium chloride standards produced a mean molar response of only 0.42% that of glycine, and this value has been used to correct measurements on the basis of their estimated ammonia content. In most cases the correction was a rather minor one.

Ammonia excretion was measured in separate metabolic experiments using the method of Solórzano (1969). Duplicate analyses were made on 5 ml samples read against distilled water blanks and ammonium chloride standards at 640 mμ on a Spectronic-20 colorimeter.

Ozone in the product water of the sea water system was monitored using "standard methods" (Taras *et al.*, 1971).

Animals

All the specimens were collected at low tide from the shallow waters of Tampa Bay, and identified according to the most authoritative published sources that could be obtained. Before use they were maintained one to three days in the laboratory in clean water of similar temperature and salinity to that from which they were obtained (approximately 34‰ and 29°C). Only healthy appearing, active animals were employed. The shells of molluscs were meticulously scrubbed with a brush, and tubiculous annelids carefully dissected from their tubes before the experiments. The hydroid, *Hydractinia*, was left attached to the shell on which it was found, and the measurements include the weight of this shell.

Metabolic measurements

Since the species used varied considerably in their weight and composition, it was necessary to find a meaningful common denominator by which they could be equated. Their metabolic rates, as measured through oxygen consumption, were chosen for this purpose. These rates were determined by placing one or more specimens in stoppered glass jars submerged in a container of purified sea water. The size of these jars was chosen so that usually within one to two hours the animals could reduce the oxygen content to about ⅔ of the initial level, at which time the experiment would be terminated. The jars also contained a rotating magnetic stirring bar protected in a small plastic cage. Small samples of water were withdrawn with a 2 ml syringe and injected into a Radiometer blood gas analyzer. The oxygen partial pressure values obtained were converted to volume measurements using solubility coefficients calculated from the formula provided by Green and Carritt (1967).

Also during these experiments, ammonia excretion rates were determined. Preliminary duplicate water samples were withdrawn from the jars just before they were sealed, and other samples taken immediately after the last oxygen measurement. The estimated excretion rates were calculated from the differences between the two sets of samples.

FAA exchange measurements

Animals, individually or in small groups, were placed in vessels scaled to their size (mostly less than one liter) and flushed with test media for 20 to 30 minutes at flow rates of several hundred ml per minute. A glass tube provided a steady stream of gentle bubbles for mixing and some aeration. An experiment was begun by simultaneously taking the first samples and removing the inflow of flushing medium. Subsequent samples were withdrawn after 2, 5, 10, 15, 20, 30, 40, and 60 minutes. (In some cases with small volumes the 2 and 15 minute samples were omitted). Each experiment was repeated three times with the same animals; first at minimal initial FAA levels, then (by adjusting the flow rate of the syringe pump) at an intermediate level estimated to be within the range of natural sea water, and finally at a level judged to be slightly higher than the natural concentration. Thus, 27 (or 21) datum points, each the mean of a duplicate analysis, were obtained to characterize the rates of FAA uptake and release by each species.

RESULTS

An example of the results of the FAA experiments may be seen in Figure 2. Each of the illustrated datum points represents the measured value less a minor correction (lower, double-dashed line) for estimated fluorescamine response due to ammonia released by the specimens (upper, dashed line).

To practically evaluate these data and meaningfully compare different animals, the curves must be reduced to simpler parameters. A relatively straightforward empirical mathematical model appears to provide the most convenient and effective procedure for doing this, rather than models based solely on enzyme kinetic theory (Ferguson, 1980a). This model assumes that the rate of uptake by the animals is dependent on the FAA concentration of the medium, and that the rate of release, within the time frame and conditions of the experiment, is constant. Thus, the change of concentration of FAA within the experimental vessel is described by:

$$\frac{dC_t}{dt} = \lambda - kC_t$$

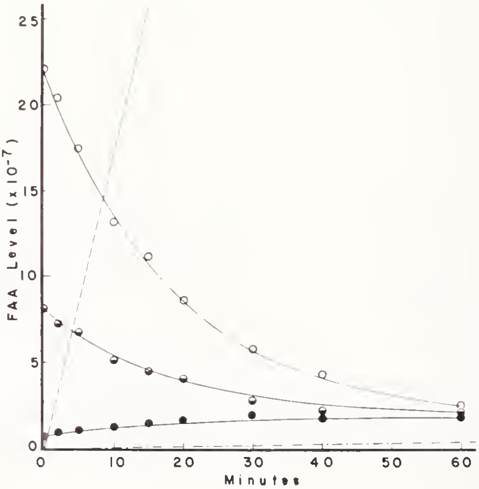

FIGURE 2. Changes in media concentration of FAA produced with three different initial levels (represented by ○, ◒, and ●) by the sea urchin, *Lytechinus variegatus* (81.0 g). The curved lines represent values predicted from the model ($k = 6.63 \times 10^{-4}$; $\lambda = 12.35 \times 10^{-11}$ moles/g/min.). Also shown is the estimated accumulation of ammonia from excretion (upper, dashed straight line), and the fluorescamine response correction based on it (lower, double-dashed straight line) (Initial fluid volume = 1025 ml).

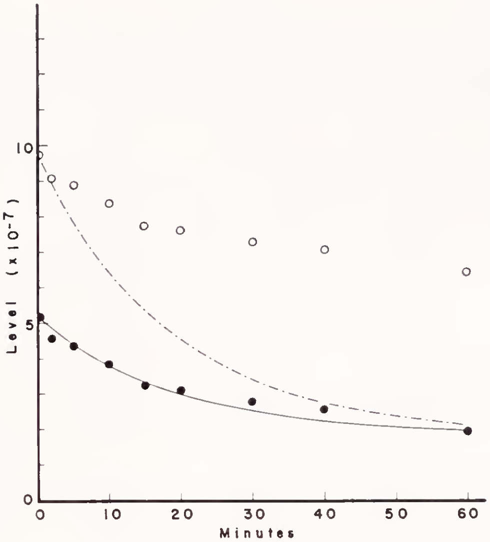

FIGURE 3. Removal of primary amines from filtered bay water by *Lytechinus*. Open symbols represent actual measured changes in concentration; closed symbols represent values less a 4.5×10^{-7} *M* correction for non-FAA reactants. Curved lines are the changes predicted by the model (same parameters, λ and k, as in Fig. 2)—dashed line with the uncorrected concentration; solid line with the corrected concentration. With the correction, a much improved fit is obtained.

where (with standardization of units),

λ = the rate of FAA efflux from the animals (moles/g/min.)

k = the fractional rate of influx (from 1 liter into 1 g/min.)

C_t = the FAA concentration (*M*) of the medium at time "t" (min.).

The working form of the equation, including corrections for specimen weight (w) (=g) and vessel fluid volume (v) (=ml) is:

$$C_t = \frac{\lambda}{k} + \left(C_0 - \frac{\lambda}{k}\right)e^{-k \cdot t \cdot w \cdot 1000/v}$$

It may be further noted that the ratio of the two constants (λ/k) corresponds to the equilibrium concentration of FAA in the medium at which the rate of influx would be just equal the rate of efflux.

The major difficulty in the practical use of this equation is that it must be solved by inspection. In the present case, this was facilitated by a computer program, which also corrected for the small volume changes induced by the withdrawal of each sample, and the ammonia effect. The values of k and λ selected were those which produced a correlation coefficient (r) closest to unity when predicted values were matched to the 27 (or 21) datum points.

The curved lines shown in Figure 2, then, represent the best fit values predicted by the model in this fashion. Table I lists the determined values for the fractional rate of influx (k), rate of efflux (λ), and related data recorded for each of the 21 species. The computed equilibrium levels (λ/k) are listed in Table II.

TABLE I

Influx and efflux of free amino acids (FAA) by various estuarine species

	Total weight	FAA influx (k)	FAA efflux (λ)	Fit (r)	NH_3 Excretion rate
Species (number)	(g)	$(\times 10^{-4})$	$(10^{-11}$ m/g/min.)		$(10^{-11}$ m/g/min.)
PORIFERA					
Cliona celata (1)	315.0	1.39	0.70	0.99176	373
CNIDARIA					
Hydractinia echinata (on shell)	10.7	5.03	14.70	0.99939*	408
Calliactis tricolor (1)	2.0	6.91	22.35	0.99712*	0
MOLLUSCA					
Fasciolaria distans (2)	71.0	1.52	4.22	0.99935	138
Melongena corona (1)	112.5	1.86	4.00	0.99827	303
Cantharus tinctus (9)	33.5	3.22	9.90	0.99861	718
Macrocallista nimbosa (1)	165.0	1.75	3.90	0.99742	42
Mercenaria campechiensis (1)	286.0	0.69	0.83	0.99949	193
Crassostrea virginica (1)	102.0	2.27	6.15	0.99642	185
Argopecten irradians (1)	21.4	11.72	15.95	0.99570	678
ANNELIDA					
Glycera americana (3)	0.8	15.43	61.00	0.99892*	933
Diopatra cuprea (9)	4.5	8.49	54.80	0.99674	2867
Cirratulus hedgepethi (2)	3.6	7.29	42.00	0.99802*	712
ARTHROPODA					
Limulus polyphemus (1)	10.9	1.34	0.00	0.99967	3090
Emerita talpoida (6)	8.0	2.97	29.60	0.99951	1200
ECHINODERMATA					
Astropecten articulatus (1)	18.5	2.76	6.90	0.99959	61
Ophiophragmus filograneus (2)	1.9	7.15	18.90	0.99785*	43
Leptosynapta crassipatina (1)	0.3	6.05	41.00	0.99981*	381
Lytechinus variegatus (1)	128.5	6.63	12.35	0.99914	833
Mellita quinquiesperforata (1)	69.0	2.56	4.75	0.99953	300
CHORDATA					
Styela plicata (1)	59.8	2.35	1.78	0.99620	417

(m = moles)

Best fit of 27 or 21 (*) points after correcting for NH_3 excretion (34‰ S, 29°C).

The values of k and λ are useful for some purposes, but since they are based on weight, they are highly affected by the relative proportion of skeleton, body fluid, and active tissue of each species. Thus, a somewhat more meaningful comparison is obtained if influx and efflux are computed in terms of metabolic rate. The measured metabolic rates and the proportional FAA fractional influx and efflux calculated in terms of them are presented in Table II.

Table II

Comparison of free amino acid influxes and effluxes per unit metabolic rate, and equilibrium levels (normal environment = 7.5 to 17 × 10^{-7} M *FAA).*

	Metabolic rate	FAA influx	FAA efflux	FAA equilibrium level	FAA sustaining level
Species	(μL O_2/g/min.)	(10^{-4} *M*/μL O_2/min.)	(10^{-11} *M*/μL O_2/min.)	(10^{-7} *M*)	(10^{-7} *M*)
PORIFERA					
Cliona celata	0.25	5.6	2.8	0.50	181
CNIDARIA					
Hydractinia echinata	0.60	8.4	24.5	2.62	123
Calliactis tricolor	0.62	11.1	36.0	3.61	93
MOLLUSCA					
Fasciolaria distans	0.60	2.5	7.0	2.77	398
Melongena corona	0.78	2.4	5.1	2.15	422
Cantharus tinctus	0.45	7.2	22.0	3.07	143
Macrocallista nimbosa	0.31	5.6	12.6	2.22	180
Mercenaria campechiensis	0.11	6.3	7.5	1.20	161
Crassostrea virginica	0.20	11.4	30.8	2.70	91
Argopecten irradians	0.97	12.1	16.4	1.36	85
ANNELIDA					
Glycera americana	1.57	9.8	38.9	3.95	106
Diopatra cuprea	2.38	3.6	23.0	6.45	287
Cirratulus hedgepethi	0.98	7.4	42.9	5.76	141
ARTHROPODA					
Limulus polyphemus	9.77	0.1	0.0	0.00	7300
Emerita talpoida	4.68	0.6	6.3	9.96	1590
ECHINODERMATA					
Astropecten articulatus	0.46	6.0	15.0	2.50	170
Ophiophragmus filograneus	0.54	13.2	35.0	2.64	79
Leptosynapta crassipatina	2.12	2.9	19.3	6.77	358
Lytechinus variegatus	0.27	24.6	45.7	1.86	43
Mellita quinquiesperforata	0.15	17.1	31.7	1.85	60
CHORDATA					
Styela plicata	0.60	3.9	3.0	0.75	257

While the values listed are characteristic of each species under the conditions that have been defined, it is particularly useful to relate them to the actual FAA levels that the animals encounter in their normal habitats, or at least, natural bay water. Therefore, samples of bay water were collected from the same vicinities as were the animals. These were immediately filtered in the field and analyzed within 30 minutes. They revealed a considerable variability and distinct differences between conditions at low tide (a few cm from the bottom) and high tide (about a meter above the bottom). The mean response of 12 low tide samples (± standard deviation) was 21.63 ± 6.40 × 10^{-7} *M*, while 13 high tide samples had a mean of 12.64 ± 2.23 × 10^{-7} *M*. Furthermore, it was found that if containers of bay water were returned to the laboratory and filtered in bulk, much lower levels still would be recorded. Six such samples had a mean of 7.98 ± 2.17 × 10^{-7} *M*. This decline was assumed to be due to the rapid removal of dissolved organics by the rich plankton content. The overall appearance is that large quantities of FAA leach out of bottom detritus and sediments into the water column and are rapidly taken up by living organisms in the water.

All these measurements were, of course, recorded in glycine equivalents. Clearly the values do not represent just FAA, but also other filterable primary amines and, especially, ammonia. While the means were not available to differentiate these components, a simple experiment was conducted to evaluate the total level of non-FAA represented.

A quantity of bay water was brought to the laboratory and filtered as quickly as possible. The specimen of *Lytechinus* previously studied was then flushed with this water and the experiment repeated using the same water as the experimental medium. The results may be seen in Figure 3. The measured levels declined, but at a rate much slower than that predicted by the model using the constants determined from defined media. However, if a value of 4.5×10^{-7} *M*, representing metabolically inert fluorescamine reactants, is subtracted from the observed values, a close fit is obtained. The same result has been reached in similar experiments with other animals.

TABLE III

Percent of metabolism supported by free amino acid in the normal range of bay water concentrations (7.5 to 17×10^{-7} M).

Species	% Metabolism gross (uptake only)	% Metabolism net (uptake − loss)
PORIFERA		
Cliona celata	4.2–9.5	3.9–9.2
CNIDARIA		
Hydractinia echinata	6.3–14.2	3.8–11.8
Calliactis tricolor	8.4–18.9	4.8–15.3
MOLLUSCA		
Fasciolaria distans	1.9–4.3	1.2–3.6
Melongena corona	1.8–4.1	1.3–3.5
Cantharus tinctus	5.4–12.2	3.2–10.0
Macrocallista nimbosa	4.2–9.6	3.0–8.3
Mercenaria campechiensis	4.7–10.7	3.9–9.9
Crassostrea virginica	8.5–19.3	5.4–16.2
Argopecten irradians	9.1–20.5	7.4–18.9
ANNELIDA		
Glycera americana	7.4–16.7	3.5–12.8
Diopatra cuprea	2.7–6.1	0.4–3.8
Cirratulus hedgepethi	5.6–12.6	1.3–8.4
ARTHROPODA		
Limulus polyphemus	0.1–0.2	0.1–0.2
Emerita talpoida	0.5–1.1	(−)0.2–0.4
ECHINODERMATA		
Astropecten articulatus	4.5–10.2	3.0–8.7
Ophiophragmus filograneus	9.9–22.5	6.4–19.0
Leptosynapta crassipatina	2.1–4.8	0.2–2.9
Lytechinus variegatus	18.4–41.7	13.8–37.2
Mellita quinquiesperforata	12.8–29.0	9.6–25.8
CHORDATA		
Styela plicata	2.9–6.7	2.6–6.4

Table IV

*Comparison of observed rates of uptake and release of FAA by a sunray clam (*Macrocallista nimbosa*) while closed and while open with siphons extended.*

	Influx (k)	Efflux (λ)	Fit (r)	Metabolic rate
	($\times 10^{-4}$)	(10^{-11} m/g/min.)		(μL O_2/g/min.)
Closed	0.02	0.17	0.99968	0.03
Open	1.75	3.90	0.99742	0.31

This correction value of 4.5×10^{-7} *M* was then subtracted from the mean values of field filtered water samples. It was thus estimated that the mean Tampa Bay FAA levels vary from approximately 7.5 to 17×10^{-7} *M*, depending largely on the state of the tide. The extreme range of observed values, corrected in this fashion, was 4.0 to 31.3×10^{-7} *M*.

Using the mean values (7.5 to 17×10^{-7} *M*), and the constants, k and λ, determined for each species, it is possible to calculate the quantity of FAA taken up and given off within this range of environmental conditions. Furthermore, since the aerobic metabolism of a mole of amino acid (molecular weight 100) requires approximately 100 liters of oxygen, an estimation may be made of just what proportion of each species' metabolism is satisfied through dissolved FAA in the water. These computations have been tabulated in Table III.

The actual environmental levels encountered by some species, especially those living in the sediments, are difficult to evaluate, but they may be much higher than those of sea water. It is useful, then, to look at what implied effects higher concentrations might have in supplying the metabolic needs of various animals. Thus, a calculation has been made (using the same equivalency of 1 mole FAA to 100 liters O_2 consumption) to estimate the environmental level that would produce net influx just providing for the total measured metabolic rate. These values, referred to as "sustaining" levels, are also listed in Table II. Their presentation is for reference, and it is not meant to be implied that the animals can (or cannot) actually sustain themselves completely at these levels solely from FAA, or that the transport systems responsible for uptake would function with the same effectiveness at these higher concentrations. Indeed, saturation of the transport systems might be expected.

Finally, one additional experiment was completed to serve to some extent as a control for the other work. Strong rubber bands were placed around a previously studied sunray clam (*Macrocallista*), effectively preventing it from pumping. The complete experimental procedure was then repeated. As expected, the observed metabolic rate and FAA fluxes were reduced to negligible levels (Table IV).

Discussion

The mathematical model used appeared to be wholly satisfactory for describing the data obtained in each experiment. The illustrated case of *Lytechinus* (Fig. 2) is fairly typical of the results obtained with the other animals. Any model, however, is a simplification of reality. The present approach has assumed a homogeneity of the amino acids taken up and released that obviously does not exist. A mixture of amino acids was used in the experiments in the belief that the data obtained by that means would be the most reflective of the unknown natural situation. The high proportion of glycine was included not only because all measurements were made against this substance as a standard, but also because previous work (Ferguson, 1971) revealed that it is by far the most significant component released by starfishes into sea water.

The success of the model in fitting the data seems to generally bear out these assumptions. It also substantiates the implication of the model that each species, for the defined conditions, has a characteristic rate of uptake that is dependent primarily on the external FAA concentration. For this to be the case, the transport systems involved must possess high affinities and saturation levels. Such properties have been reported by other investigators, but their demonstration may be affected by the experimental conditions (*e.g.*, Wright and Stephens, 1977; Wright *et al.*, 1980). Since the model used in the present work is empirical, it makes allowance not only for the kinetic properties of the various transport systems, but also for the physical limitations of movement of media over the absorptive surface, and possible other biological characteristics of each species.

The model also implies a constant release rate from the organisms. Alternative models have been examined which allow the intracellular pools to increase with uptake, and thus induce greater efflux. These more complex models appeared to offer little or no advantage in matching the data, and they were abandoned. The intracellular amino acid pools seemed to remain rather stable and scarcely affected by the range of substrates employed in these experiments. Such stability could be due to their large size or to rapid metabolic interconversions within the cells.

Clearly there are major differences between the species in their characteristic fractional rates of influx and rates of efflux (Table I). Many of these differences must relate to the various physical conformations of the animals. For example, the southern quahog clam, *Mercenaria*, has low recorded rates, but most of its weight is represented by its especially massive shell. The errant polychaete, *Glycera*, on the other hand, has extremely high rates, but completely lacks a hard skeleton. There probably is no fully satisfactory way to evaluate the differences of such disparate species, but a number of calculations included in Tables II and III are helpful.

It can be seen immediately in these that only arthropods fail to carry on meaningful exchanges. This must be related to the presence of an exoskeleton covering all their exposed surfaces, and it was a basic finding of Stephens and Schinske (1961). In the present cases, minor values have been recorded, especially for the small and very active mole crabs (*Emerita*). This may possibly be due to epiflora, as Anderson and Stephens (1969) have suggested in studies with several small arthropod species. Bacterial uptake of hexoses has also been described by Castille and Lawrence (1979) in work with post larval penaeid shrimp. If the values listed here do reflect ingestion and excretion, these processes can only represent very minor proportions of the animals' total metabolic activities.

While some contribution by epiflora cannot be completely ruled out in other cases, the effect must be almost negligible. The animals were all clean and well flushed with nearly sterile media before the experiments, and the levels of organics used were not such as to encourage a rapid bloom of bacteria within the short time frames studied. Cultures of wipes taken from animals and vessels failed to reveal large amounts of bacterial growth and, in other work, antibiotics have not significantly inhibited uptake. Autoradiographs (Ferguson, 1967) have shown that uptake is into the epidermal layer, and not into particles attached on the surface. Furthermore, the control experiment with the bound specimen of *Macrocallista* (Table IV) is not indicative of a significant bacterial contribution in comparison to the normal exchanges that take place.

All of the species other than arthropods to demonstrate significant levels of uptake and net uptake, as well as specific differences. The equilibrium levels (Table II) are all low, and definitely less than the mean minimum FAA level (7.5×10^{-7} *M*) found in the waters from which the animals come. This confirms the hypothesis that soft-bodied marine animals do indeed gain net sustenance through the removal of dissolved organics from sea water, and the existence of this process should now be assumed to be universal for all such species, unless specific proof is acquired to the contrary. The only substantial report claiming the reverse process, that marine animals suffer net loss of amino acids, is that of Johannes *et al.* (1969) on the commensal flatworm, *Bdelloura.* As the newer methods appear to be considerably more sensitive and reliable than theirs, it would be appropriate for this species to be re-examined.

It is significant that mechanisms providing for net uptake of FAA are at least as well developed in sponges as in chordates. They exist as commonly in the lower phyla as in the higher ones. Thus, they must have evolved very early, probably in some form even before any of these phyla were differentiated. They possibly represent the most ancient means by which animals obtain their nutrition. While they have been lost with the development of impervious exoskeletons, as in the arthropods, their apparent universal retention by other invertebrate species attests to their continuing significance in the biology of these forms. Most of the differences in the values of the parameters listed in Tables I, II, and III, thus, appear to relate to specific evolutionary adaptations in life styles, and not to unique differences that developed phylogenetically.

A comparison of the influx and efflux rates in Table I shows that when one rate is high there is a tendency for the other to be high also and *vice versa*, and this is partly responsible for the consistency of the low equilibrium levels (Table II). Higher paired rates are especially evident for *Calliactis, Crassostrea, Argopecten, Ophiophragmus, Lytechinus,* and *Mellita.* Thus, there seems to be some kind of connection between the two. Most likely, influx and efflux occur in the same tissues, and the correlation reflects a measure of the amount of the metabolically active tissues that are exposed to efficient ventilation.

While the equilibrium levels for all the soft-bodied species are low, those of some are slightly higher than others. This is especially the case for the burrowing tubiculous annelids and, interestingly, the burrowing holothuroid, *Leptosynapta.* Stephens (1975), Costolpulos *et al.* (1979), and others have speculated that burrowing annelids can obtain significant FAA from high levels that appear to exist in the sediments, but since the animals normally live in tubular chambers formed from various types of secretions, it is uncertain just how much advantage they can take of this source, and their true relationship may be more with the water at the sediment-water interface. In the present study it was found that the near bottom water (low tide) itself contains significantly more FAA than that higher in the water column. So, the worms most certainly must be exposed to somewhat elevated FAA levels. Their higher equilibrium values appear to reflect an adaptation to these levels.

The molluscs as a group make for some interesting comparisons. In the case of the predacious gastropods, *Fasciolaria* and *Melongena*, benefit from exogenous uptake is limited to several percent of the metabolic rate (Table III). Presumably much of this uptake is located in the gills, as much of the rest of the body is covered by shell. While it is a small quantity, it may be sufficient to supply the energy needs of the ciliated cells that maintain the water flow over these organs to sustain respiratory exchange.

In contrast, the bay scallop, *Argopecten*, exhibits a much greater metabolic dependence on FAA. This species not only has large gills in relation to its body size, it also has numerous pallial tentacles and an extensive area to its mantle. Thus, its adaptations include a rather large ventilated surface area and a reduced mass. Its relatively high dependence on FAA may be a factor in the restriction of its normal habitat to presumably richer bay and nearshore waters, especially on grass beds. The oyster, *Crassostrea*, while possessing a very different life style, appears to share some of these same properties.

Heavier, burrowing bivalves, such as *Macrocallista* and *Mercenaria*, obtain benefits from net uptake at a level somewhat intermediate between the predacious gastropods and the filibranchs. As filter feeders they need large gills and efficient pumping, but they appear to sustain their larger body masses more completely through ingested food. Again, their intermediate levels of uptake may largely serve the energy needs of the ciliary pumping process.

Other interesting cases of adaptations are seen in the echinoderms. *Lytechinus* has the highest net uptake, supporting up to 37% of its metabolic rate at the 17×10^{-7} M environmental level. This species normally hides itself under shells and weeds it picks up with its many long and active tube feet. While this behavior itself might have nutritional significance in enhancing the FAA levels in the vicinity of the animals, the high level of dependence definitely correlates with the activity of the tube feet and other appendages. Autoradiographs (Ferguson, 1967, and others) have shown that these structures are especially efficient in taking up dissolved organics. Thus again, the metabolic activities of the superficial structures appear to be maintained in very large part through the direct assimilation of nutrients from the environment. Also, sea urchins often pump water through their digestive tracts, presumably to assist in respiration, and uptake might occur internally *via* this route (*cf.*, Ferguson, 1969; Pearse and Pearse, 1973).

The sand dollar, *Mellita*, and the brittlestar, *Ophiophragmus*, also exhibit a very high utilization and metabolic dependence on FAA. Both these species live in the upper levels of sandy sediments and detritus—the former near high energy beaches, the latter on grass flats. Unlike the worms, their naked bodies and motility would fully expose their surfaces to the higher levels of FAA that undoubtedly occur in these substrates. Therefore, they must have even larger proportions of metabolic dependence on this nutrient source than is indicated by Table III. Especially intriguing are the low sustaining levels (Table II) calculated for both of these species. These are well within the natural substrate levels that could reasonably be expected to be encountered. Thus, it is highly probable that exogenous uptake of dissolved organics, and not feeding, actually represents the primary nutritional source of both these species. A similar conclusion was reached for the sand dollar, *Dendraster*, by Stephens *et al.* (1978).

While exogenous uptake of FAA may be the major nutritional source of these last two forms, it is undoubtedly not an exclusive one. There is no evidence that these or any of the other animals examined in this study can survive without feeding, and certainly evolution towards different feeding strategies has been a predominant feature of most metazoans. On the other hand, it is also apparent that these and other species have undergone considerable evolutionary adaptation to enhance the benefits they can derive from exogenous uptake mechanisms. Recognition of this fact can perhaps greatly assist in understanding the peculiar structural complexities of many types. The short spineous surface texture of sand dollars and brittlestars, the frilled mantle of some molluscs, and similar kinds of ornamentation on other soft-bodied species may all be adaptations to facilitate epidermal assimilation of dissolved organic substances. The pogonophorans, lacking a gut, may as a phylum be even more highly adapted towards this source of nutrition (*cf.*, Southward *et al.*, 1979). Relatively little work has yet been completed on the exotic structural forms frequently found in the meroplankton, but this nutritional mechanism may be especially significant for larva, particularly those that utilize ciliary forms of locomotion (*cf.*, North, 1975; Fankboner and DeBurgh, 1978; Rice *et al.*, 1980).

The fact that most animals have retained mechanisms for both exogenous uptake of dissolved nutrients as well as feeding appears to indicate that each serves significant functions that are not readily provided by the other. That is, the two mechanisms are not so much redundant as complimentary. The levels of net utilization of FAA reported here, commonly less than 10% of the total metabolic needs, conforms with the previously developed hypothesis (Ferguson, 1967, 1970, 1980a,b) that this uptake generally supplies the nutritional needs of the epidermis and active superficial structures. Physiologically, this would appear to have at least two benefits. First, it would provide nutrition to parts of the body which are far removed from internal reservoirs. This process must have much greater facility than those associated with food collection, digestion, assimilation, storage, and translocation. It can provide energy directly to isolated parts, which in some cases (ciliary appendages, gills, tube feet, *etc.*) may have large energy demands. As an essentially continuous process of high efficiency, it is particularly effective in supporting metabolic activities which also tend to be continuous. Second, it assists in providing for the isolation of the internal environment from the external one. The natural levels of FAA are often orders of magnitude less than those maintained in the body of fluids of most marine animals (*cf.* Awapara, 1962). Clearly, for unprotected species these gradients create problems, especially if large quantities of metabolites must be conducted to the epidermal tissues to support ciliary movement, secretion, and other forms of work. If superficial tissues can obtain most of their sustenance directly from the environment, permeability barriers may develop to help retain these metabolites. Certainly the data reported here show that losses of FAA are very low indeed, and even these residual losses are more than compensated for by the uptake mechanisms.

It is concluded, then, that direct uptake of FAA (and probably other dissolved organic nutrients) plays a vital role in the biology of essentially all soft-bodied marine invertebrate species, especially serving to support epidermal functions and metabolite retention. Furthermore, a number of species have become specifically adapted to utilize this form of sustenance as a major source of nutrition with it in some cases providing more benefits than ingested food. Thus, the importance of these mechanisms should not be overlooked in either physiological or ecological investigations of marine animals.

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EFFECTS OF TEMPERATURE EXTREMES ON PROTEIN SYNTHESIS IN LIVER OF TOADFISH, *OPSANUS TAU, IN VIVO*

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Abstract

Amino acid incorporation and polypeptide chain elongation rates were determined in toadfish at the upper and lower ends of their range of temperature tolerance. The method was based on pulse injection of radioactive amino acids into the hepatic portal vein and analysis of ribosome-bound and completed chains at various times after injection. Elongation rates at low temperatures were obtained from the rate of completion and release of polypeptide chains pre-labeled at 20°C. Average polypeptide chain assembly time at 4°C was 6 h; that at 37°C was 1 min. Comparison with rates of total protein synthesis obtained in previous studies indicate a coordinated slowing of all protein synthetic reactions at low temperatures. A 10-fold decline in elongation rate from 7°C to 4°C suggests a specific temperature sensitivity in elongation factors or in formation of aminoacyl-tRNA. At high temperatures (32°–40°C) protein synthetic reactions show a loss of coordination, with elongation rate increasing normally (Q_{10} about 2) while amino acid incorporation declines. The results indicate a close correlation between behavior of the protein synthetic system and observations on temperature tolerance and winter dormancy in this species.

Introduction

The toadfish is a highly adaptable marine fish that is common along the Atlantic coast from the Gulf of Maine to Cuba. Its broad temperature tolerance and hardiness in the laboratory have made it a valuable organism for examination of temperature effects on biological processes *in vivo*. Previous studies on liver protein synthesis in 20°C-acclimated toadfish *in vivo* have indicated three types of response to direct temperature change (Mathews and Haschemeyer, 1978). From about 17°C to 30°C, rates of polypeptide chain elongation and of total amino acid incorporation (measuring both initiation and elongation) show a normal Q_{10} of about 2.5. (Q_{10} is the ratio of rate at $T + 10°C$ to that at T). From 17°C down to about 7°C, Q_{10} for both processes is approximately 5. With further temperature reduction, incorporation rate declines with an even greater Q_{10}, and elongation rate can no longer be measured conveniently.

The present work examines in more detail the behavior of this system at both low and high temperature extremes. An experimental method has been used that permits effects on polypeptide elongation reactions to be distinguished from those on chain initiation or ribosome recruitment. This is of particular interest because of the loss of coordination among these reactions observed at temperature extremes in all other organisms studied. In *E. coli*, for example, initiation of protein synthesis

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is strongly inhibited at temperatures below 8°C, whereas elongation continues at a relatively normal rate (Das and Goldstein, 1968; Friedman *et al.*, 1971). This causes a run-off of ribosomes from the messenger and consequent disaggregation of the protein synthetic apparatus. A similar phenomenon occurs at high temperatures (42°–43°C) in bacterial and mammalian systems (McCormick and Penman, 1969; Patterson and Gillespie, 1972; Oleinick, 1979).

Previous studies on elongation rate and ribosome run-off have depended on analysis of polyribosome profiles. This has not been found to be a reliable method for toadfish liver because of degradation by endogenous ribonuclease (Haschemeyer, 1969a). The direct method of labeling the growing polypeptide chains and following their rate of completion and release (Haschemeyer, 1969b) is not suitable when both the incorporation of radioactive amino acids and their uptake by the tissues are significantly reduced (Persell and Haschemeyer, 1980). We have therefore adopted the approach of pre-labeling the ribosome-bound polypeptide chains by hepatic portal vein injection at 20°C, then measuring the release of these chains after fish are cooled to the experimental temperature. For experiments at high temperatures the standard pulse injection method (Mathews and Haschemeyer, 1978) has been used.

MATERIALS AND METHODS

Collection and maintenance of specimens

Opsanus tau adults, 300–400 g, were obtained from local collectors at the Marine Biological Laboratory, Woods Hole, Massachusetts, and were maintained during the summer in running seawater aquaria at 20° ± 1°C. Fish were fed chopped baitfish on alternate days; food was withheld for two days prior to experiment. For experiments at 4° ± 1°C, fish were kept in well-aerated 20 gallon aquaria in the coldroom. Fish acclimated to 10°C were kept in refrigerated running seawater aquaria for three weeks prior to use.

Experimental procedure at 20°C

Fish acclimated for at least two weeks at 20° ± 1°C were lightly anesthetized by brief exposure to 0.5 g/l ethyl-p-aminobenzoate (benzocaine) in well-aerated seawater at 20°C. The fish were transferred to a surgical rack and oxygenated seawater (without anesthetic) was circulated over the gills. The fish showed active operculation throughout the subsequent procedure. A small abdominal incision was made to expose the hepatic portal vein, and 0.1 ml of 15 m*M* (2 μCi) L-[^{14}C(U)]leucine and 2 μCi [^{3}H(G)]inulin (New England Nuclear Corp.) in balanced salts was slowly injected into the vein through a 30-ga needle (Haschemeyer, 1973). The presence of inulin served as a marker for the passage of the injection bolus through the liver (Persell and Haschemeyer, 1976). During injection, mixing of the injection solution with the portal blood was monitored visually.

For incubation times of 1 to 4 minutes, the needle was kept in the vein to prevent bleeding, after which the liver and blood draining from the liver at the hepatic vein were collected. For experiments of longer than 5 minutes duration the needle was removed, bleeding was stopped with gauze, and the abdominal incision was closed with wound clips. The fish was then quickly aroused by running 20°C seawater over the gills and returned to 20°C aquaria for a free-swimming period prior to collection of the liver. The procedure was standardized so that the time elapsed from the beginning of the injection until arousal was 5 ± 0.5 minutes.

Temperature transfer experiments

Fish acclimated at 20° ± 1°C were injected at 20°C and aroused after 5 minutes, as described above. They were immediately transferred to a container of seawater at 4°C and then to a 20 gallon aquarium in the coldroom. The rate of cooling was monitored with a rectal temperature probe connected to a Yellow Springs Telethermometer. Temperature was observed to decline with an approximately exponential dependency; cooling half-time (time required for a temperature change equal to one-half the final ΔT) was 5–7 minutes. All fish reached a temperature of 4°–5°C, as registered by the probe, within 30 minutes. Fish were then maintained at this temperature for varying periods up to 24 hours, after which liver and blood were collected.

Two groups of fish were subjected to a second temperature transfer as follows: After injection at 20°C and cooling to 4°C, as described above, they were maintained at 4°C for 1.5 to 16 hours, then returned to running seawater aquaria at 20°C. Livers were collected after 5 to 20 minutes exposure to the higher temperature. Another group of 20°-acclimated fish was cooled to 4°C without injection, maintained at that temperature for 24 hours, then returned to 20°C aquaria. After a 30 min equilibration period they were injected at 20°C for analysis of elongation rate and fractional incorporation at that temperature.

High temperature experiments

Fish acclimated at 20°C were adjusted to a temperature of 30°–32°C for 15 minutes and then anesthetized by brief exposure to 0.5 g/l benzocaine at that temperature. Each fish was further warmed on the surgical rack by a rapid circulation of heated seawater through the mouth and gills. An abdominal incision was made, and temperature was monitored with a thermometer placed under the liver. After a warming period of about 5 minutes, hepatic portal vein injection was performed as above except that a concentration of 0.1 mM ^{14}C-leucine was used. The liver was excised and hepatic blood was collected 30–60 seconds after injection.

Sample preparation and analysis

Livers were analyzed for levels of free ^{14}C and ^{3}H in the tissue and for incorporation of ^{14}C-leucine into protein (Haschemeyer and Persell, 1973). Blood collected from the severed hepatic vein was also analyzed for both isotopes. All data were converted to μCi per g liver or per ml plasma and divided by the μCi injected to yield fraction of dose recovered in the various compartments. Incorporation into protein is presented as total percent of injected ^{14}C-leucine recovered in protein-bound form or as fractional incorporation rate (fraction of dose incorporated divided by incubation time), as described by Mathews and Haschemeyer (1978). Uptake of ^{14}C-leucine into intracellular space was obtained from total free radioactivity in liver after subtraction of a small fraction associated with extracellular space. The latter was estimated by use of the inulin recovery in liver and the ^{14}C-amino acid/^{3}H-inulin ratio in hepatic blood plasma (Persell and Haschemeyer, 1976).

For determination of polypeptide chain assembly time incorporation into the total liver homogenate (T) was compared with that in the ribosome-free supernatant (S) obtained after centrifugation of the homogenate for two hours at 100,000 × g. Homogenates were first treated with sodium deoxycholate at 0.5% to dissolve membranes. Radioactive incorporation was measured on filter discs (Haschemeyer, 1969b) in a toluene-based scintillation fluid at 70% efficiency.

Calculations

For determination of average polypeptide chain assembly time (t_c), the ratio of radioactivity in soluble protein (S) to total protein (T) of liver was plotted *vs.* time. The linear portion of the curve (up to S/T = 0.5) was analyzed to obtain t_c (Haschemeyer, 1969b). Elongation rate (residues per second) was calculated from t_c by use of the number-average molecular weight (45,000) of newly-synthesized polypeptide chains in toadfish liver, as follows:

$$\text{amino acid residues/sec} = (45{,}000/115)/60t_c \qquad (1)$$

where 115 is the average residue weight in protein (Mathews and Haschemeyer, 1978).

RESULTS

Control experiments at 20°C

These experiments were run to test the validity of the modified system for determination of elongation rate *in vivo*. The method, as originally developed, depends upon the measurement of the distribution of labeled polypeptide chains between ribosomes (R) and the soluble compartment (S). The early linear dependency of S/T on time, where $T = R + S$, yields average polypeptide chain assembly time (Haschemeyer, 1969b). This may be combined with number-average molecular weight to obtain elongation rate in residues/sec (Eqn. 1). The time dependency, however, is influenced by the molecular weight distribution of chains synthesized; at later times weight-average molecular weight has a greater influence on S/T (Mathews and Haschemeyer, 1976). Use of these equations also requires a constant specific radioactivity during the experimental period. To better approximate this condition over the time periods required for temperature transfer, a higher concentration (15 mM) of the radioactive amino acid was used.

Figure 1 (left) presents the S/T results obtained in 20°-acclimated fish at 20°C using the 15 mM injection dose. The early linear portion yields a value of 5.5 min for t_c (average polypeptide chain assembly time), corresponding to an elongation rate from Eqn. (1) of 1.2 residues/sec. A similar experiment was run at 20° with

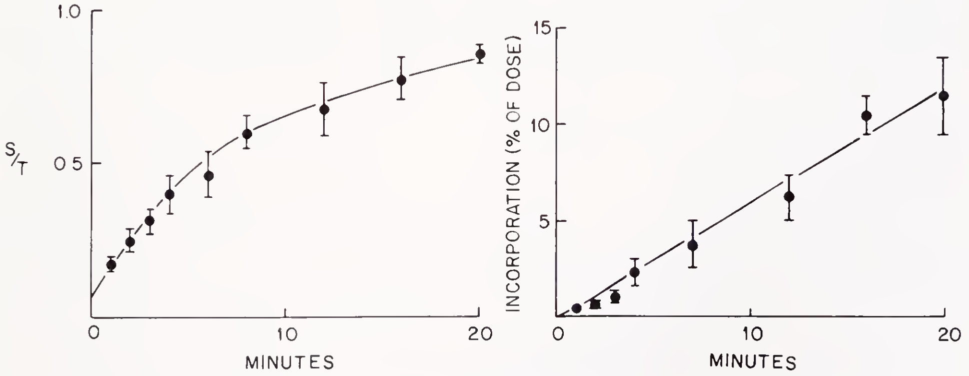

FIGURE 1. Protein synthetic parameters for toadfish liver *in vivo* at 20°C. Left: Ratio of incorporation into soluble protein (S) to total soluble protein + ribosome-bound chains (T) as a function of time after hepatic portal vein injection of 15 mM ^{14}C-leucine. Right: Incorporation into protein as % of injected dose. Animals were 20°C-acclimated toadfish, 370 ± 80 g body weight, % liver/body weight = 2.3 ± 0.5, number of fish = 30. Bars express standard deviation.

fish acclimated at 10°C for three weeks; t_c was 3.4 min, yielding an elongation rate of 1.9 residues/sec. Both values are in good agreement with results obtained at tracer or plasma leucine (0.1 m*M*) concentrations (Haschemeyer, 1969b; Mathews and Haschemeyer, 1978). At longer times S/T is observed to rise slowly toward 1.0. This behavior is consistent with theoretical prediction (Mathews and Haschemeyer, 1976) for a system in which continued incorporation of radioactivity into growing chains is occurring. Similar experiments with tracer doses yielded a more rapid rise of S/T to unity associated with a declining free radioactivity in the tissue.

The time course of incorporation of ^{14}C-leucine into protein at the 15 m*M* dose is shown on the right in Figure 1. The linearity over a period of 20 minutes indicates relatively constant specific radioactivity in the intracellular leucine pool. Incorporation rate in 20°-acclimated fish was 0.6% of the injected dose per minute; that in 10°-acclimated fish measured at 20°C was 1.2% per minute. Total recovery of radioactive leucine in liver (free + protein-bound) averaged 20% of dose; this is about one-half the level found after injection of 0.1 m*M* ^{14}C-leucine. Previous studies have indicated that declining fractional uptake with concentration is associated with saturation of the leucine transport system (Persell and Haschemeyer, 1976).

Polypeptide chain elongation after transfer to 4°C

The object of these experiments was to determine whether elongation of polypeptide chains continues in toadfish liver at very low temperatures. Previous work had indicated a large drop in incorporation of radioactive leucine into protein at temperatures below 7°C (Mathews and Haschemeyer, 1978). If this was due to a failure in initiation as in *E. coli* discussed above, ribosome run-off would occur leading to a reduction in concentration of active protein synthetic units. Conversely, the decline in protein synthesis might be due to a slowed rate of elongation without loss of active ribosomes. The time dependency of the system at 20°C described above indicated that it should be possible to label ribosome-bound polypeptide chains at 20°C and cool the animal before complete release of labeled chains had occurred.

Figure 2 presents the results for levels of incorporation of ^{14}C-leucine into protein, as % of dose, in fish labeled at 20°C and transferred to 4°C. These levels primarily reflect incorporation occurring during the 5 minute period at 20°C (about 2.5% of dose from Fig. 1) plus that taking place during cooling. Protein synthesis in this period would be expected to follow the Q_{10} = 5 dependency previously found for this temperature range (Mathews and Haschemeyer, 1978). The results at 0.75–

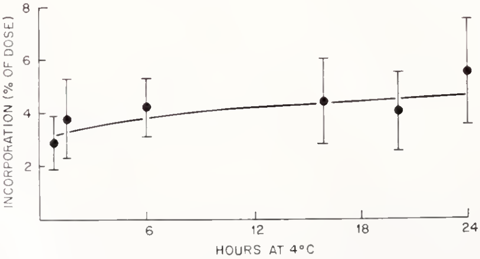

FIGURE 2. Incorporation levels in fish labeled at 20°C and transferred to 4°C. The recovery of ^{14}C-leucine in protein is expressed as % of dose administered. Bars indicate standard deviation; number of fish = 44.

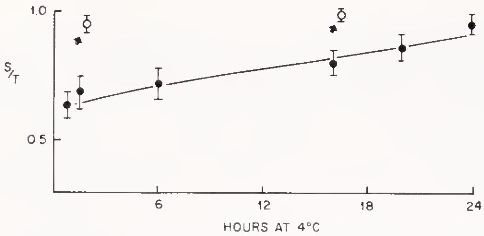

FIGURE 3. Time dependency of release of pre-labeled polypeptide chains at 4°C. The ratio of incorporation into soluble protein (S) to total soluble protein + ribosome-bound chains (T) is plotted as a function of time at 4°C. Closed circles: fish maintained at 4°C until analysis; open circles: fish re-warmed to 20°C after the indicated time at 4°C. Bars express standard deviation; N = 4–6 at each time point.

1.5 hours after injection (see Fig. 2) thus include both of these contributions. Further incorporation at 4°C is clearly very low (0.1% of dose per hour) and is obscured by individual variation. Such a low rate is consistent with data obtained when ^{14}C-leucine is injected directly at 4°C (Mathews and Haschemeyer, 1978). Measurements of free radioactivity indicate that ^{14}C-leucine is available for incorporation throughout this period, although declining from 20% of dose at t = 5 min to 7% at 24 h.

Figure 3 presents the S/T data for the fish transferred to 4°C. Individual variation here is much less than in Figure 2 because S/T is less dependent on amino acid uptake and specific radioactivity of the intracellular pool than are S and T separately. These data clearly show a continuous slow rise in S/T following the pre-labeling and cooling period. Most significantly, there is no evidence of the rapid approach to unity expected if elongation proceeds normally while initiation is inhibited.

In order to test the reversibility of the profound slowing of elongation indicated by the data of Figure 3, two groups of fish were transferred to 4°C and equilibrated for 1.5 or 16 h, then returned to 20°C water. The open circles in Figure 3 indicated by arrows represent average values of S/T found for these animals after 10–20 minutes of 20°C exposure. Values at 5 min were somewhat less. These results are consistent with resumption of elongation at a normal rate, leading to completion and release of the pre-labelled chains. The possibility that the protein synthetic system simply fell apart as a result of these temperature changes was not supported by the results in another control experiment. Fish were exposed to 4°C for 24 h without prior injection, re-warmed to 20°C as above, then analyzed in an experiment comparable to that of Figure 1. The data indicated normal protein synthetic behavior: incorporation rate was 0.75% of dose/min (±0.2%; N = 7); average polypeptide chain assembly time was 4.7 minutes. These results do not differ significantly from those in the fish not subjected to temperature change (Fig. 1).

The S/T data of Figure 3 may be analyzed by comparison with a theoretical curve where S/T is 0.5 after one round of protein synthesis, 0.75 after two rounds, and so on (Haschemeyer, 1969b). This, however, does not take into account heterogeneity in chain size which affects the S/T profile, particulary at $S/T > 0.5$ (Mathews and Haschemeyer, 1976). The problem is simplified by reference to the control data at 20°C (Fig. 1) where average chain assembly time is known. On this basis, the change in S/T profile observed during the time period of 4 to 24 hours at 4°C is equivalent to 3.2 rounds of protein synthesis. This yields an average chain assembly time at 4°C of 6 hours; elongation rate is 0.018 residue/second.

Effects of elevated temperatures

These experiments were designed to test the ability of the liver protein synthetic system to maintain coordination during acute exposure to high temperatures. Fish acclimated at 20°C were first brought to 30°C for about 15 min, then rapidly warmed with heated seawater to temperatures ranging from 32°C to 40°C. The speed of protein synthesis was such that it was necessary to reduce incubation times to 30–60 sec in order to stay within the linear range of S/T. The S/T results yielded continuously increasing values for elongation rate with temperature (Q_{10} about 2), as shown in Figure 4. This behavior is similar to that observed in mammalian cells in culture, although the possibility of premature termination of chains under these conditions cannot be ruled out. Average polypeptide chain assembly time at 37°C was 1 minute, as in rat liver (Mathews and Haschemeyer, 1976).

Fractional incorporation, reflecting both elongation rate and active ribosome concentration, however, declined rapidly at temperatures above 32°C. The Arrhenius plot (Fig. 4, closed circles) indicated a Q_{10} of about 1/40 or activation energy of −290 kJ/mole. This fall in incorporation did not appear to be a consequence of reduced uptake of the radioactive amino acid. Levels of intracellular ^{14}C-

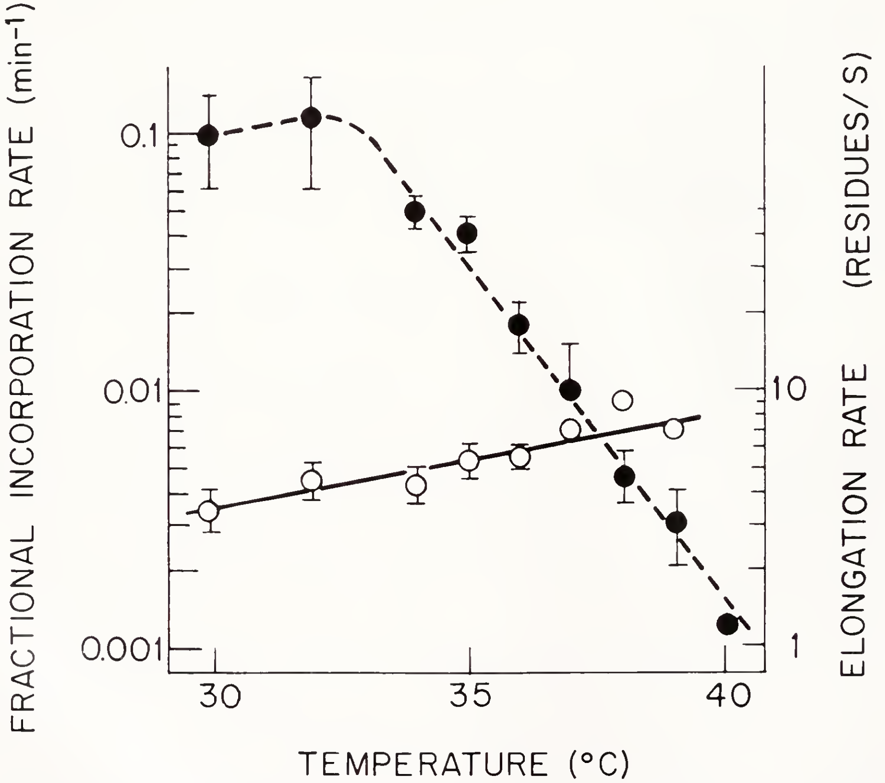

FIGURE 4. Arrhenius plot for toadfish liver protein synthesis at high temperatures. The fractional rate of incorporation of ^{14}C-leucine into protein (closed circles, left scale) and elongation rate (open circles, right scale) are given as a function of body temperature. Exposure time at each temperature was about 5 minutes. Rates are given on a logarithmic scale; bars indicate standard deviation; number of fish = 30.

leucine at 1 min after injection averaged 25% of dose from 30° to 38°C, close to levels observed at more moderate temperatures (Persell and Haschemeyer, 1976). Above 38°C, uptake fell to 15% of dose, suggestive of membrane dysfunction.

Fish that were warmed to a body temperature of 37°C, then returned to 30°C, showed normal protein synthetic parameters at 30°C. Fish exposed to higher temperatures (up to 43°C) exhibited only partial recovery (10–25%) of incorporation rate when returned to 30°C. Effects on the central nervous system also were evident at the higher temperatures. Partial jaw clamping occurred at 36°–38°C; tight clamping and body stiffening were observed at 39°–43°C. However, all fish maintained operculation and gave evidence of normal circulation, based on inulin movement through the liver. None died during the short periods of these experiments, although tests using one-hour exposures indicated a lethal temperature of about 35°C.

DISCUSSION

The experiments presented above represent the first detailed analysis of protein synthetic parameters at extreme temperatures in a vertebrate liver *in vivo.* Previous attempts to characterize this system in toadfish at very low temperatures were limited by the low levels of uptake of radioactive amino acids and of incorporation into protein (Mathews and Haschemeyer, 1978). The method presented here permits determination of elongation rate from the rate of completion of pre-labeled polypeptide chains. Hepatic portal vein injection was used to obtain rapid uptake and labeling of chains. A more constant specific radioactivity was achieved by the use of 15 m*M* leucine in place of the usual tracer or plasma concentration. This helps to minimize differences in pool size as well as time-dependent changes in specific radioactivity caused by turnover. Pool swamping has been used effectively for measurements of protein synthesis in mammals (see, *e.g.*, McNurlan and Garlick, 1980). Studies in toadfish indicate that the 15 m*M* leucine injection does not affect liver protein synthesis as measured by incorporation of another amino acid (Persell and Haschemeyer, 1976).

The results of the temperature transfer experiments indicate that elongation of chains proceeds very slowly at 4°C, at a rate of about 1/10th that previously observed at 7°C. The effect accounts for the observed change in total protein synthesis in this temperature range, as measured by direct amino acid incorporation (Mathews and Haschemeyer, 1978). There is thus no indication of a specific blockage of initiation leading to ribosome run-off in this system. Rather, the response is evidently a slowing down of all steps without loss of coordination among chain initiation, elongation, and release reactions. Under these conditions the system is able to shift up immediately in response to increased temperature, as evidenced by the re-warming experiments.

The properties described above are consistent with the ability of the toadfish to adapt to widely fluctuating environmental temperatures and with its dormancy in winter (Gudger, 1908). The molecular basis for the large depression in elongation rate, however, is not clear. Measurements of aminoacyl-tRNA binding by elongation factor 1 do not give evidence of any unusual temperature sensitivity (Plant *et al.*, 1977). The synthesis of the precursor aminoacyl-tRNA, however, could be affected, and is currently under study. Protein synthetic rates may also be influenced by membrane transitions in this temperature range. Another factor that may play a role in the toadfish system is the requirement for adjustment of blood pH with temperature change (Rahn and Baumgardner, 1972).

The behavior of the toadfish system at temperatures approaching the upper lethal limit resembles that seen in other organisms. Total protein synthesis reached its maximal rate at 32°C, then deteriorated rapidly as temperature was increased. Elongation and release of chains, however, showed an apparently normal increase in rate up to 38°C. This would support a specific effect on initiation reactions, as suggested for other systems (McCormick and Penman, 1969; Patterson and Gillespie, 1972; Oleinick, 1979). A failure of initiation complex formation occurs *in vitro* at the same temperatures found to inhibit protein synthesis in whole cells (Mizuno, 1975; Bonanou-Tzedaki *et al.*, 1978). These temperatures, however, approach the upper limit of membrane integrity (see, *e.g.* , Janoff *et al.*, 1979, for *E. coli*). In the toadfish system the changes in protein synthesis were observed at temperatures well below those at which ^{14}C-leucine uptake by liver was affected. The activation energy for inhibition was similar to that observed for protein denaturation (Johnson *et al.*, 1974). Although not conclusive, these results would support the view that denaturation of a specific protein synthetic component may be a primary event, as opposed to general membrane dysfunction. Further studies in isolated cells may be helpful in distinguishing these effects. Also of interest is the possibility that disassembly of the protein synthetic system at high temperatures is associated with a shift to production of heat shock proteins, as in other organisms (Lemaux *et al.*, 1978; Moran *et al.*, 1978).

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FEEDING PATTERNS OF THE LONG-FINNED SQUID, *LOLIGO PEALEI,* IN NEW ENGLAND WATERS

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ABSTRACT

Gut content analyses have shown that the diet of the long-finned squid, *Loligo pealei*, differs between inshore spawning and nursery grounds and offshore winter grounds. In this study, squid were collected inshore from May through November in lower Narragansett Bay, Rhode Island and offshore during winter along the continental shelf between Cape Hatteras and Cap Cod. In both collections crustaceans were more frequently consumed than either fish or squid, but fish were eaten by a wider size range of squid and more frequently inshore. Prey-type selection based on size was common in both samples, but it is unlikely that the species composition is the same in both areas. These data suggest that *L. pealei* is a highly opportunistic predator, whose diet primarily reflects the local abundance of potential prey species. Such a flexible feeding strategy could account for the large spatial and temporal variations which have been reported in the diet of this squid from various offshore areas.

INTRODUCTION

Studies of the trophic dynamics of the California squid, *Loligo opalescens* (Morejohn *et al.*, 1978), clearly indicate the central role of this intermediate predator in coastal food webs. Squid are important because they both compete with and serve as prey for higher carnivores, and thus must be carefully considered when developing multispecies fishery management strategies (May *et al.*, 1979). Comprehensive studies of the trophic dynamics of *Loligo pealei*, the common squid of New England, have not yet been reported. In spite of the fact that *L. pealei* is an important food source for many commercially important fish, both inshore and offshore (Verrill, 1882; Tibbetts, 1975, 1977), details of its own feeding dynamics while inshore are generally lacking. However, weakfish (*Cynoscion regalis*) and scup (*Stenotomus crysops*) have been reported in the stomachs of *L. pealei* (Oviatt and Nixon, 1973) from Narragansett Bay, and squid have been directly observed feeding upon menhaden and sculpin (*Brevoortia tyrannis* and *Myxocephalus* sp., H. W. Pratt, NMFS, personal communication), sand lances (*Ammodytes americanus*, Caroline Griswold, NMFS, personal communication), silversides, mummichogs, anchovies, and grass shrimp (*Menidia menidia, Fundulus* spp., *Anchoa mitchili*, and *Palaemonetes* spp., author).

Studies of the offshore feeding habits of *Loligo pealei* (Vovk, 1972, 1974; Vinogradov and Noskov, 1979) indicate that feeding activity varies diurnally and seasonally, and that the relative importance of crustaceans, fish, and other squid in the diet varies with the size of the squid. The broad latitudinal and depth ranges of this species may be expected to be reflected in the diets of squid from different areas as well. *L. pealei* is continuously distributed from the coast of Columbia to

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Nova Scotia (Cohen, 1976). In the northern half of its range at least (Cape Hatteras through New England), *L. pealei* overwinters on the continental shelf and slope at depths to over 200 m (Summers, 1969; Vovk, 1978) and migrates in late spring and early summer into shallow coastal spawning areas (Verrill, 1882; Haefner, 1964; Summers, 1968, 1971; Macy, 1980).

The goals of this study were therefore to first determine the composition of the inshore diet of *Loligo pealei*, and to then interpret the results in light of our current understanding of the basic life history patterns and strategies of this and other similar squid species.

MATERIALS AND METHODS

Details of the sampling procedure and sampling dates will be briefly outlined here since they have been reported elsewhere (Macy, 1980, 1982). Samples of approximately 100 squid each were collected at two-week intervals from late April through November, 1978 (inshore samples) in lower Narragansett Bay, Rhode Island, U. S. A. Three additional samples, taken during early and late winter (R/V Cryos, 1976 and R/V Argus, 1977, 1978) on the continental shelf (offshore samples), were also provided by the National Marine Fisheries Service, Woods Hole, Massachusetts. Since specialized knowledge is required to taxonomically classify prey items on the basis of the small fragments found in squid stomachs (Bidder, 1950; Karpov and Cailliet, 1978), squid gut contents were only grossly categorized on the basis of appearance as "crustaceans", "squid", "fish", "algae", "unknown", and combinations of the first three groups. Since all samples were taken without regards to time during the daylight hours, generally in mid-morning for the inshore samples, no attempts were made to correlate feeding patterns with time of day. Mantle length, sex, and stage of maturity were recorded, however, to allow analysis of possible sex or size related food preferences. Insights into the actual feeding behavior were obtained from direct observations of squid under natural and laboratory conditions.

RESULTS

Slightly over 50% of inshore and offshore squid had empty guts (Table I), but no obvious relationship between size of the squid (mantle length, ML) and the presence or absence of food could be detected. The extreme ranges of mantle lengths were similar in both collections, from 1–34 cm inshore (Fig. 1a) and 3–29 cm offshore (Fig. 2a), but the modal sizes differed considerably. The modal length of the inshore samples was about 5 cm, primarily reflecting the presence of large numbers of young of the year, while offshore squid were larger with a modal length of about 10 cm. With respect to prey composition, crustaceans, squid, and fish accounted for over 80% of the total stomach contents in both collections (81.9% and 86.3%, Table I), on a frequency of occurrence basis. While the relative importance of crustaceans in the diet was also similar between samples, 58% inshore and 49% offshore (Table I), the importance of fish and squid did differ. Inshore, fish ranked second in importance at 17%, followed by squid in 11% of the guts. In the offshore samples, squid ranked second at 20%, with fish third at 12%. Unidentified matter amounted to about 10% in both samples, and in four inshore squid small amounts of green algae were found. Since squid are carnivores, the incidence of plant matter in the guts must be considered spurious (see Vovk, 1972, and Stunkard, 1977 for other records of algae).

TABLE I

Stomach contents by frequency of occurrence of prey in pooled 1976 R/V Cryos and 1977–78 R/V Argus (offshore) and 1978 Narragansett Bay (inshore collections). Of a total of 3875 inshore squid, 2667 were examined for stomach contents. F = fish; C = crustaceans; S = squid.

	Offshore		Inshore	
Food	Frequency	% With food	Frequency	% With food
Empty	465		1419	
Crustaceans	191	49.48	724	58.01
Squid	79	20.47	139	11.14
Fish	46	11.92	214	17.15
F & C	13	3.37	16	1.28
S & C	8	2.07	16	1.28
S & F	5	1.30	13	1.04
Algae	0	0.0	4	0.32
Unknown	44	11.40	122	9.78
Total	851	100.01	2667	100.00
With Food	386	45.4	1248	46.79

The data presented in Figures 1b and 2b also show that food type varies with the size of the squid. Crustaceans were most frequently eaten by squid 9 cm ML or smaller in the inshore samples and by those less than 14 cm ML from offshore. Squid and fish both seem to be eaten by all but the smallest squid from both areas, but were the dominant food (present in greater than 50% of the food-containing guts) in only the larger squid. In the inshore samples (Fig. 1b) fish became most important in squid 11 cm and larger (60% of those with food), but in the offshore samples (Fig. 2b), squid or fish were not dominant until squid reached sizes of 16 cm (66.7%) and larger. There also appears to be a tendency for those squid from offshore to feed less on a single food type than do their inshore counterparts. The narrow peaks of fish and squid consumption in both collections (Fig. 1b and 2b) are artifacts of small sample size in the larger squid size classes.

Observations of feeding activities and behavior of wild and captive squid (Macy, 1980) provided another source of information. Long-term observations of captive squid revealed that a behavior pattern described by Williams (1909, cited by Bidder, 1950) and Stevenson (1934) in conjunction with feeding and spawning activities, respectively, is actually quite common at all times, particularly during periods of bright illumination. The behavior seems to represent a resting posture, in which the squid lie on the bottom with their posterior ends and tips of the arms in contact with the substrate, when resting, waiting for prey, and eating. Typically the squid also assume a transversely banded cryptic coloration pattern, which blends well with a mottled substrate of rocks, pebbles, sand, and algae. Wild squid have been frequently seen resting on the bottom, and on occasion they have been seen dropping to the bottom to avoid large predators such as striped bass (*Morone saxatilis*) (H. W. Pratt, NMFS, Narragansett, personal communication). With regards to the prey of squid, adults 15 cm ML and larger have been observed feeding eagerly on small shrimp (*Palaemonetes* and *Crangon*) which they picked individually from the bottom, as well as on much larger prey such as menhaden nearly one-half their own length (see Macy, 1980, Fig. 34).

Prey attributes other than size alone may be important also. In the laboratory, spiny fish such as sticklebacks (*Apeltes* and *Pungitius*) were captured but rejected,

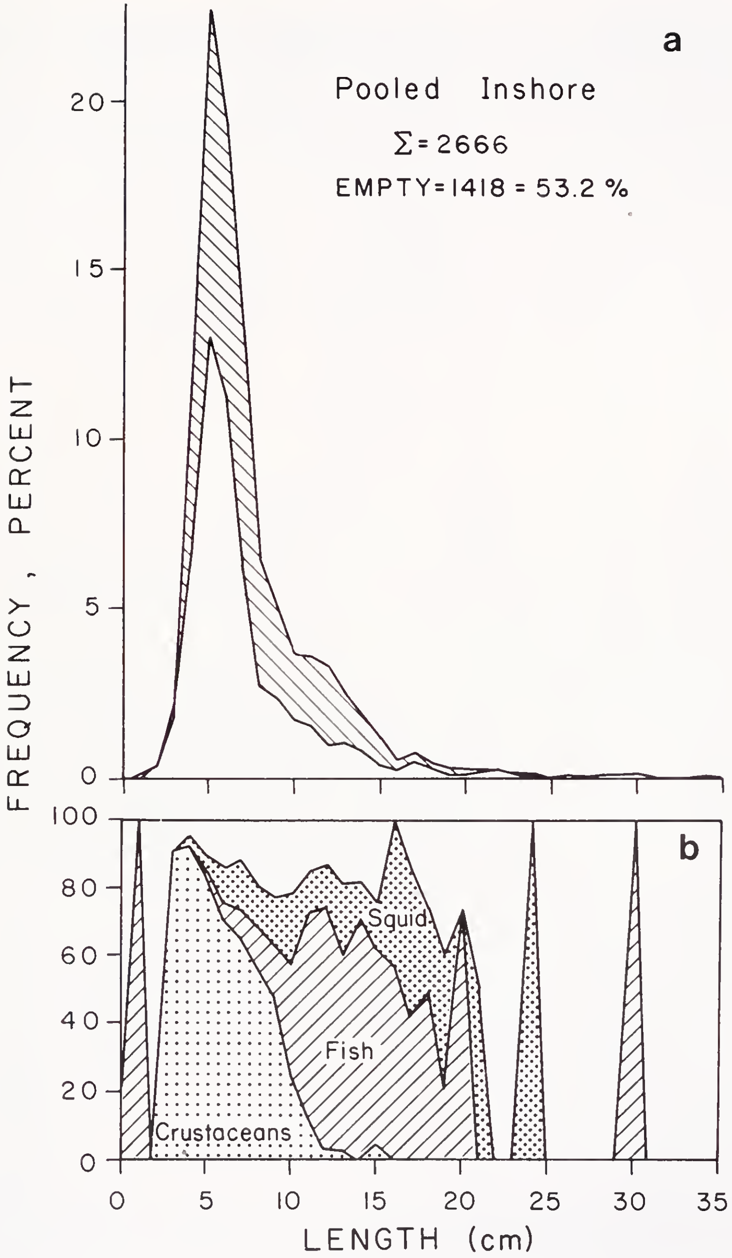

FIGURE 1. Size distribution of squid containing food (cross-hatching in a.) and the distribution of major prey categories as a function of the size of squid (b) from the pooled Narragansett Bay collections. The unhatched area in (a) represents squid without food.

while other species such as striped mummichogs (*Fundulus majalis*) appeared to be difficult to successfully subdue. When fed individuals of this species larger than about 50 mm fork length, even large squid (greater than 15 cm ML) frequently

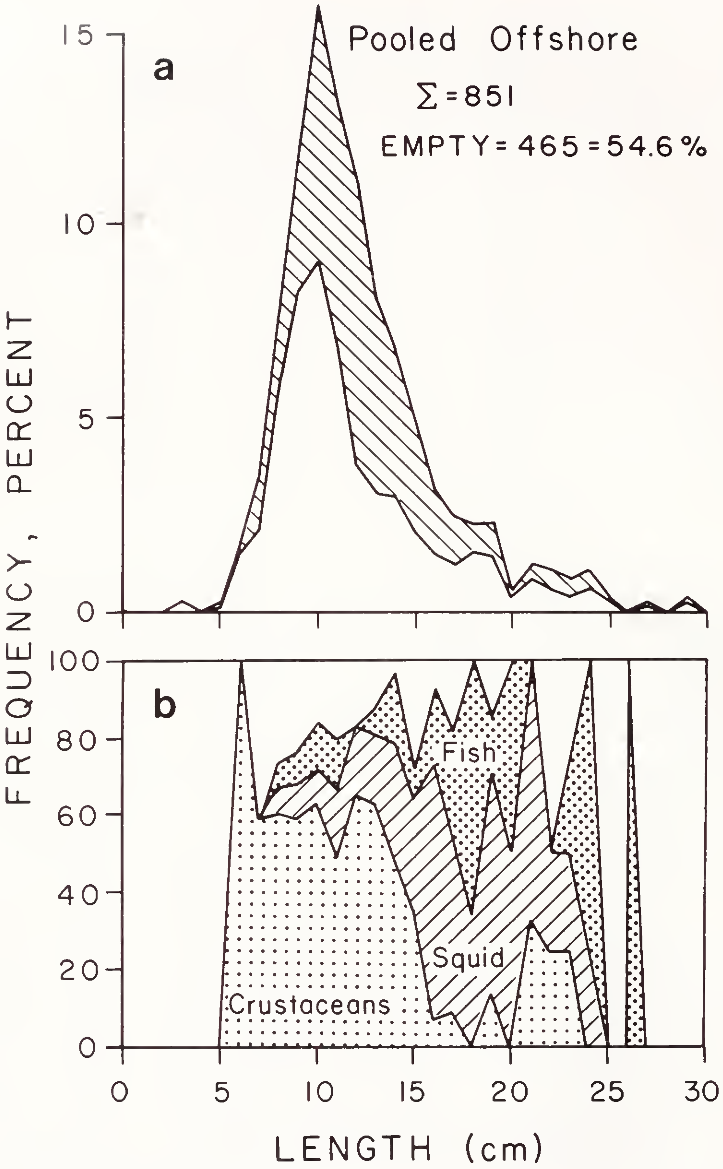

FIGURE 2. Size distribution of squid containing food (a) and the distribution of major prey categories as a function of the size of the squid (b) from the pooled offshore collections.

damaged the tips of their arms and tentacles. Aside from such problem fish, captive squid successfully attacked and ate a wide variety of fish of the genera *Anchoa, Menidia, Fundulus, Cyprinodon, Ammodytes, Brevoortia,* and even *Anguilla,* provided that they were no larger than perhaps 30% of the squid's mantle length.

Parasitism of squid by the tetraphyllidean cestode, *Phyllobothrium lologinis* Linton, 1907, whose intermediate host is thought to be a copepod (Stunkard, 1977) was evident in the early summer inshore samples. Most frequently the parasites were observed in the caeca and stomachs of the squid, but occasionally individuals were found unattached in the mantle cavity or emerging from the anus. Typically 1–2 parasites were observed per squid (up to 6 on occasion), but only the larger squid were infected: 2 parasitized squid less than 10 cm mantle length were seen. A clear seasonal pattern of infection was evident. In the May 31 sample, 40% of the squid were infected, but the rate had fallen to between 18 and 21% during the period between June 30 and July 17. By late July less than 5% of the inshore samples contained parasites, and rates remained practically nil for the remainder of the inshore season. No parasites were observed in the 102 squid from the November 1977 R/V Argus offshore sample, but 9 of the 151 squid from the March 1978 R/V Argus sample, all larger than 12 cm ML, were infected.

DISCUSSION

Relative prey size

In general, the diets of inshore and offshore squid do not appear to differ greatly with respect to their gross prey composition. Crustaceans were found in 50% or more of the guts from both samples, and were nearly twice as important overall as fish and squid combined. Before these results can be compared with those of other studies, however, two points should be noted. First, various methods have been used to evaluate the importance of particular prey (Table II), and second, the size distribution of squid has not always been adequately accounted for. The latter point is especially important when comparing the diets of squid from different areas or seasons (Table II). The offshore data from this study ("pooled lengths," Table II) agree well with those of Vovk (1972) on a frequency of occurrence basis, but values expressed on a volume percent basis suggest that fish were more important overall than crustaceans. On a weight percent basis (Vinogradov and Noskov, 1979, Table II), fish appear to be still more important.

Food preferences based upon the relative sizes of predator and prey have been previously reported for *L. pealei* (Vovk, 1972), as well as for *L. opalescens* (Fields, 1965; Karpov and Cailliet, 1978) and *Illex illecebrosus* (Squires, 1957; Ennis and Collins, 1979; Vinogradov and Noskov, 1979; Amaratunga, 1980), and would be expected considering certain morphological changes which occur as squid grow. In loliginids at least, streamlining improves with growth (*i.e.,* increased length:width ratios) (Haefner, 1964; Macy, 1980), which allows higher pursuit speeds to be attained (Packard, 1969). Relative fin area also increases with length, which enhances mobility. In practice, however, the range of suitably-sized prey for a given squid appears to be quite large. Adult *L. pealei* have been observed eating small shrimp perhaps only one-twentieth of their mantle length, while Vovk (1972) found that fish as large as 19 cm may also be eaten. O'Dor *et al.* (1980) suggests that *I. illecebrosus* can successfully attack and subdue fish equal to their own mantle length. At the population level, both inshore and offshore samples revealed abrupt changes in prey preference as the squid grow. Inshore, the transition between crustaceans and fish occurred at about 9 cm mantle length, while offshore the shift from crustaceans to a mixed fish/squid diet occurred at about 15 cm mantle length. These findings also agree with those reported by Vovk (1972) for offshore areas. Size constraints are likely to be particularly important to hatchlings and young juveniles, however, because of their limited speed and mobility. Unfortunately, gut analyses for very small individuals are lacking at present.

TABLE II

Relative importance of prey items in the diet of Loligo, *based on gut content analyses.Legend: M.L. = dorsal mantle length; N.E. Shelf = New England continental shelf, Cape Hatteras to Cape Cod unless otherwise noted; Euph = euphausids; Cop = copepods.*

Location, month collected (source)	M.L., cm		*Crustaceans*	*Fish*	*Squid*
				% frequency	
Loligo pealei					
Narragansett Bay, V–IX (this study)	pooled		58.0	17.2	11.1
	3–15		59.0	16.4	10.5
	16–30		0.0	41.2	35.3
N.E. Shelf, XI–XII, III (this study)	pooled		49.5	11.9	20.5
	3–15		56.7	8.9	16.0
	16–30		10.0	45.0	28.3
X (Vovk, 1972)	pooled		44.9	21.1	26.6
				% total volume	
X? (Vovk, 1972)	pooled		37.1	38.4	18.6
				% total weight	
N.E. Shelf, VIII–IX (Vinogradov and Noskov, 1979)	pooled		20.4	53.4	26.2
Georges Bank, VIII	8–15		100.0	—	—
	16–30		65.0	32.0	3.0
Nantucket, VIII	8–15		55.0	13.0	32.0
So. of Long Island, IX	8–15		33.0	39.0	28.0
	16–30		17.0	47.0	36.0
Loligo opalescens			*"index of relative importance"*		
Monterey Bay, I–XII (Karpov and Cailliet, 1978)		*Euph.*	*Cop.*	*Fish*	*Squid*
Deep Water	pooled	6552.2	103.8	1.3	2.6
Shallow Water	pooled	1553.4	1.0	44.8	187.6
Spawning Grounds	pooled	0.0	0.0	11.3	0.0
Overall	2.1–10	3988.0	97.5	2.0	16.2
	10.1–18	5400.0	37.5	8.8	9.7

Prey availability

The wide geographic range of *L. pealei* should be reflected in the varying importance of given prey types in the diets of comparable sized squid from different locations. In the Narragansett Bay samples (Fig. 1b) squid of all sizes tended to feed on a single prey type, and few squid larger than 10 cm ML contained crustacean remains. In the offshore samples, however, a mixed diet was more evident (Fig. 2b) in the 12–25 cm size classes, and crustaceans were present in squid of all sizes. In August on Georges Bank (Vinogradov and Noskov, 1979) (Table II) 8–15 cm squid fed exclusively on crustaceans, while during September, south of Long Island, New York, the same size squid were feeding about equally on crustaceans, fish, and squid. In the colder, deeper waters euphausids are an important food for *L. pealei* (Vovk, 1972; Vinogradov and Noskov, 1979), as they also are for *L. opalescens* (Karpov and Cailliet, 1978) and *Illex illecebrosus* (Squires, 1957; Ennis and Collins, 1979; Amaratunga, 1980). Copepods, hyperiid amphipods, and mysid and pandalid shrimp may also be important. Myctophid fishes are heavily preyed upon in offshore waters (Vovk, 1972; Vinogradov and Noskov, 1979), but especially during fall large squid also feed on small silver hake (*Merluccius bilinearis*), while small squid and hake alike compete for fish, squid, shrimp, and euphausid resources (Vovk, 1975; Vinogradov and Noskov, 1979).

One may thus expect significant changes in the diet and perhaps even the feeding behavior to occur when squid move into the shallow inshore environment. The Narragansett Bay area is a particularly rich nursery ground for a wide variety of fish, many of which are abundant during the summer months when the squid are present (Tracy, 1905; Richards, 1963; Jeffries, 1968; Oviatt and Nixon, 1973; Saila and Pratt, 1973; Jeffries and Johnson, 1974). It is not, therefore, surprising that fish formed the bulk of the diet of squid as small as 10 cm there. Offshore, relatively large pelagic crustaceans such as euphausids and copepods (*Meganyctiphanes, Candacia, Calanus,* and *Centropages*) were the dominant prey of squid as large as 10–15 cm ML (Vovk, 1972; Vinogradov and Noskov, 1979), but these organisms are not generally abundant in Narragansett Bay. According to Jeffries and Johnson (1973), the local inshore zooplankton is dominated by relatively small copepods such as *Acartia, Temora, Pseudocalanus,* and *Centropages*. It is also evident from the incidence pattern of the copepod-carried parasite described previously (*e.g.* only large squid from offshore infected) that the unidentified host copepod must not be present in inshore waters either. Pelagic shrimps and other crustaceans may be replaced in importance inshore by demersal forms such as *Palaemonetes* and *Crangon* (Zinn, 1969; Oviatt and Nixon, 1973) and by a variety of larval and juvenile crabs which are also present (Hillman, 1964; Reilly, 1975; Oviatt and Nixon, 1973). Thus the availability of a large juvenile fish resource coupled with a lack of large pelagic crustaceans inshore can reasonably account for the prevalence of fish in the diets of a wider size range of squid than was seen offshore.

Loliginids have been considered to be demersal squids (Bidder, 1968; Zuev and Nesis, 1971; Packard, 1972), but it appears that *Loligo pealei*, in particular, may be more closely associated with the benthos while inshore than had been realized. Offshore it remains on or near the bottom during the day (Serchuk and Rathjen, 1974), but disperses into the water column at night, apparently to feed upon diurnally migrating lanternfishes, euphausids, and mysids (Vovk, 1972; Vinogradov and Noskov, 1979). Peak feeding activity, however, is thought to occur during the day (Vovk, 1972), but benthic or demersal prey are not common in their guts (*op. cit.*). Locally, *L. pealei* has been observed feeding day and night and at the surface or on or near the bottom. Typically adult squid are seen hovering, catching and eating prey, and resting on or near the bottom, and there is some evidence that even hatchlings and small juveniles remain near the bottom particularly during the day (Raytheon, 1978). Gut content analyses which could confirm this hypothesis are lacking for *L. pealei*, but it has been shown that *L. opalescens* does shift to a benthic feeding pattern when it moves onto the Monterey Bay spawning grounds (Karpov and Cailliet, 1978) (Table II).

Feeding and movement patterns

The high incidence of empty guts from inshore (about 53%) is somewhat surprising considering the high metabolic activity of the squid and the rapid growth of young of the year during the summer months. Studies of loliginids indicate that digestion of a meal takes only 2–6 hours (Bidder, 1950; Karpov and Cailliet, 1978; Macy, unpublished), and food consumption rates are also high. Vinogradov and Noskov (1979) estimated that the daily ration of *L. pealei* is only 3.2–3.8% wet weight d^{-1}, but laboratory studies (Macy, 1980) have suggested considerably higher rates of 9–10% d^{-1}, which are comparable to those reported for *L. opalescens* (Karpov and Cailliet, 1978). During the first 6 months after hatching, juvenile squid are thought to grow from 1.0 to over 2.0 cm mo^{-1} (Summers, 1968, 1971; Mesnil, 1977). In light of these data, food supplies must be abundant. Stomach fullness peaks between 1600 and 2000 h daily, and during the summer 90% or more squid may be expected to have food in their guts (Vovk, 1972). Thus inshore feeding activity was probably greatly underestimated in this study because samples were generally taken before noon, prior to highest feeding activity.

During the winter months when squid concentrate in canyon mouths along the continental slope (Summers, 1969; Serchuk and Rathjen, 1974), food supplies may become limiting. Although the overall incidence of squid with empty guts from offshore was only slightly higher than from inshore (55% *vs.* 53%) in the present study, values ranged from 49% to 68% for individual collections within the pooled sample (*e.g.* Cryos 1976, Argus 1977, 1978), which are consistent with other published results. Vovk (1972), for example, found that the incidence of empty guts typically exceeds 60% from November through March offshore, and even during the August-November period, values as high as 54% were reported from the New England continental shelf (Vinogradov and Noskov, 1979). Winter growth rates of only 0.4–0.6 cm mo^{-1} (Mesnil, 1977), presumably reflect lack of food, and it should also be noted that in late spring and early summer even immature juveniles leave the slope waters and move onto "feeding grounds" (Vovk, 1978) further inshore on the shelf.

Loligo pealei is clearly an opportunistic predator, whose highly mobile fish-like mode of existence allows it to effectively utilize a wide variety of potential prey species. In highly productive coastal waters such as Narragansett Bay and Vineyard Sound (Summers, 1968), food resources are plentiful. In the slope regions where squid overwinter, productivity is probably considerably lower. No significant energy storage reserves (other than gonads) have been found in this species either, and thus frequent feeding may be required. Since squid tend to concentrate in the relatively warmer canyon mouths, prey "patch" size and the spacing between patches may be the most important factors which determine whether or not minimum daily metabolic needs are met. Much of the variability in the reported incidence of empty guts offshore must reflect inherent variability (patchiness) in the distribution of prey species. High levels of cannibalism, mainly among larger squid (Table II), may also indicate lack of other suitable prey within an area. Thus, the annual inshore-offshore migrations which *L. pealei* makes north of Cape Hatteras may be more closely related to the need to insure adequate food supplies for reproduction and growth of young than to avoidance of excessive ranges of temperature or salinity (Hixon, 1980; Whittaker, 1980). Temporary local movements into salt ponds and canals, as have been observed are clearly food-oriented.

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THE REVERSIBILITY OF SUBITANEOUS AND DIAPAUSE EGG PRODUCTION BY INDIVIDUAL FEMALES OF *LABIDOCERA AESTIVA* (COPEPODA: CALANOIDA)

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Abstract

In Vineyard Sound, Massachusetts the calanoid copepod, *Labidocera aestiva* undergoes a period of facultative diapause that is induced by short daylength photoperiods. This study examines egg production by individual females. It is shown that some, if not all, females can switch the type(s) of egg produced. The results indicate that in Vineyard Sound females adjust to the decreasing daylengths experienced during the fall by switching production from subitaneous to diapause eggs. I suggest that a female is triggered to switch the type of eggs produced after perceiving a specific number of short daylength photoperiods. The conversion is not instantaneous, however, and during this transition phase a female produces both subitaneous and diapause eggs. It is suggested that the reproductive capacity of the population is enhanced by this type of flexible diapause response.

Introduction

The marine calanoid copepod, *Labidocera aestiva* undergoes a period of facultative diapause in Vineyard Sound, MA (Grice and Gibson, 1975; Marcus, 1979, 1980). Adults of *L. aestiva* are in the plankton from June through mid-December, during which time females produce subitaneous eggs. These eggs hatch within 2–3 days at 20.0°C. Diapause egg production commences in early September, gradually increases throughout the fall, and is accompanied by a decrease in subitaneous egg production. The diapause eggs overwinter on the sea-bottom and hatch the following spring. The production of subitaneous and diapause eggs is regulated by a temperature compensated photoperiodic response (Marcus, 1980, 1982).

Previous investigations of proportionate changes in subitaneous and diapause egg production of *L. aestiva* have been based on eggs obtained from groups of females. This study examines egg production by individuals to determine whether the type(s) of eggs produced by a female is fixed or reversible. The results indicate that *L. aestiva* females can switch the type of egg produced as a result of the photoperiodic regimen experienced by the mature animal. The impact of this flexibility on the population biology and evolution of *L. aestiva* is discussed.

Materials and Methods

Egg production by individuals before acclimation

Labidocera aestiva adults were collected from June 1979 through December 1979 from a 1–2 m depth in Vineyard Sound by towing a 1 m, 243μ mesh net for 10 minute periods. Adult females were removed from the tow samples by pipette

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and placed individually into separate 100 ml dishes containing filtered seawater (glass fiber). The dinoflagellate, *Gymnodinium nelsoni*, was added as food (5×10^2 cells/ml). The dishes then were covered and placed overnight in an incubator at 19.0°C, with a 12L:12D photoperiod. The following morning, eggs produced by each female were counted and transferred to another set of dishes containing clean filtered seawater. The identification of eggs as subitaneous, diapause, or non-viable was determined by the protocol described by Marcus (1979, 1980).

Egg production by individuals after acclimation to antagonistic photoperiodic regimens for 1 to 2 weeks

The procedures for maintaining and rearing *L. aestiva* in the laboratory described by Marcus (1980) were followed in this study, using the same schedule for feeding and transfer of adults to clean filtered seawater. Each day that eggs were collected from a carboy the type(s) of eggs produced were identified using samples of 100–120 eggs of the total number collected, according to the protocol of Marcus (1980).

A preliminary experiment (Table I, #19) used 25 females and 25 males reared to reproductive maturity at 13.5°–15.5°C and 8L:16D in the laboratory. At the onset of egg production the photoperiodic regimen was altered to 12L:12D. The

TABLE I

Temporal changes in the percentage of subitaneous, diapause and non-viable eggs produced in each carboy following exposure to an antagonistic photoperiodic regimen.

Experiment	Antagonistic photoperiodic regimen	Day	Percent subitaneous	Percent diapause	Percent non-viable
19	12L:12D	2	14	66	20
		5	23	64	13
		7	27	61	12
		9	38	35	27
		12	44	51	5
		14	43	48	8
		16	43	44	13
33	8L:16D	1	90	0	10
		11	75	15	10
		13	65	32	3
34	8L:16D	1	91	0	9
		6	62	25	13
		15	43	37	20
		19	4	80	16
		21	27	42	31
36	8L:16D	1	84	0	16
		6	21	70	9
		10	14	83	3
		12	8	85	7
41	18L:6D	1	0	100	0
		11	39	55	6
		13	35	26	39
		15	92	—	—
		17	93	—	—

temperature regimen was not changed. The type(s) of eggs produced after the change was monitored every 2–3 days for 16 days.

Next, in three experiments (Table I, #s 33, 34, and 36) begun 5 August, 14 August, and 5 September 1980, adult copepods were collected from Vineyard Sound according to the procedure outlined above and the type(s) of eggs produced overnight by isolated females was ascertained. During the few days needed to determine the type(s) of eggs produced by each female, males collected at the same time as the females were maintained in 1800 ml beakers containing filtered seawater at 19.0°C, and 8L:16D, and fed *Gymnodinium nelsoni.* Females that produced mostly subitaneous eggs as part of the first overnight clutch (*i.e.,* no diapause eggs, but some non-viable eggs) then were placed with the males in a 19 l glass carboy containing filtered seawater, and dinoflagellates (*Gymnodinium nelsoni*, *Gonyaulax polyedra*, *Prorocentrum micans*, and *Peridinium trochoideum*). Ten to 20 adults of each sex were used for each experiment. The carboy was mounted on a mechanical rotator (Marcus, 1980), in an incubator set at 17.0°–19.0°C and 8L:16D. Thereafter, the type(s) of eggs produced in each carboy was monitored every few days for a minimum of 12 days.

A fourth experiment (Table I, #41) used adults collected in October 1980. The procedure was as described above except that females producing mostly diapause eggs (*i.e.,* no subitaneous, but some non-viable) were used, and conditions in the incubator were 17.0–19.0°C and 18L:6D.

RESULTS

Egg production prior to acclimation

During 19 June–13 August 1979, the type(s) of eggs produced by individuals was determined for 70 females collected from Vineyard Sound. Most eggs produced by these females hatched within 4–5 days, and hence were classified as subitaneous. Eggs that did not hatch within 4–5 days were identified as non-viable according to the criteria of Marcus (1979, 1980). No diapause eggs were obtained.

Diapause eggs were first obtained on 27 August 1979. Their production persisted until 12 December 1979, when collecting was terminated because adults could no longer be obtained in Vineyard Sound. During this period, 64 of the females collected, isolated, and studied as individuals produced no eggs after 1 night of incubation. The initial (within 4–5 days) hatch of eggs at 25.0°C varied among the 131 remaining females. For 76 no eggs hatched within 4–5 days. For 20, more than 80% hatched. For the remaining 35, less than 80% hatched. The unhatched eggs of 73 females were chilled at 5.0°C for a minimum of 30 days, and then warmed at 25.0°C to determine if they were diapause or non-viable eggs. Of these females, 25 produced heterogeneous (*i.e.,* consisting of both subitaneous and diapause eggs) overnight clutches, 45 produced homogeneous (*i.e.,* subitaneous or diapause eggs) overnight clutches, and 3 produced non-viable eggs.

Egg production after acclimation

Table I shows the results of the laboratory acclimation study. The percentages of subitaneous and diapause eggs changed in each experiment after the photoperiodic regimen was altered. In the preliminary experiment (#19) subitaneous egg production was low (14%) when the animals were first exposed to 12L:12D. Subsequently the percentage of subitaneous eggs produced increased gradually and stabilized at 43% after 2 weeks of exposure to 13.5°–15.5°C and 12L:12D.

Experiments #33, 34, 36, and 41 indicate that the type(s) of eggs produced by a female change in response to a change in photoperiodic regimen. In experiments 33, 34, and 36 females that initially produced only subitaneous eggs were maintained with males under photoperiodic conditions (8L:16D) that normally induce production of diapause eggs (Marcus, 1980, 1982). Within 1–2 weeks some or all of the females produced diapause eggs. The percentage of subitaneous eggs in each carboy declined, and the percentage of diapause eggs increased as acclimation continued. Animals collected later in the year (*e.g.,* September, experiment 36) responded to the change more quickly than those obtained earlier (*e.g.,* August, experiment 33). For example, after 10 days at 17.0°–19.0°C and 8L:16D, females in experiment 36 produced only 14% subitaneous eggs, whereas after 11 days at the same conditions, females in experiment 33 still produced 75% subitaneous eggs.

Experiment 41 indicates that females producing diapause eggs can switch and produce subitaneous eggs in response to an appropriate change in photoperiod. This change also requires 1–2 weeks.

DISCUSSION

This investigation has shown that the diapause response of *Labidocera aestiva* is reversible. Adult females collected from Vineyard Sound, MA produced clutches of subitaneous and/or diapause eggs during their first 24 h in the laboratory. These clutches indicate the type(s) of eggs such females would normally have produced in the field. The females that produced both types of eggs may be programmed to always produce heterogeneous clutches. On the other hand, mixed clutches probably represent a transition phase indicating that a female is switching from subitaneous to diapause egg production.

The laboratory acclimation experiments clearly show that some, if not all, adult females can switch the type(s) of eggs they produce. In experiments 33, 34, and 36 egg production was not monitored for all individual females. But since all females were observed to produce only subitaneous eggs initially, some or all must have changed their egg production, as evidenced by the appearance of diapause eggs. The same is true of experiment 41 for which the switch occurred in the reverse direction.

Females require 1 to 2 weeks to adjust. The best example of this is experiment 19 since the entire history of the test animals is known. When the photoperiod was changed from 8L:16D to 12L:12D the percentage of subitaneous eggs gradually increased from 14% to 43% over the period of 12 days. These equilibrium values for subitaneous egg production are comparable to the 13.2% and 49.0% obtained by Marcus (1980, 1982) for experimental populations reared continuously at 8L:16D and 12L:12D, respectively. As for some insects (Gibbs, 1975; see Beck, 1980), the induction of diapause in *L. aestiva* may be determined by a cumulative input of short daylength light-dark cycles. If females possess different response thresholds (*i.e.,* they differ with respect to the number of cycles required to effect the change) due to individual genetic variation, then this would explain the gradual change, followed by stabilization, observed in experiment 19 after the photoperiod was changed. The results of experiments 33, 34, and 36 further substantiate the cumulative light-dark cycle hypothesis. The copepods collected in September (experiment 36) must have experienced more short daylength cycles than those collected in August (experiments 33 and 34) and as predicted by the hypothesis they responded more quickly to the antagonistic photoperiodic conditions experienced in the laboratory.

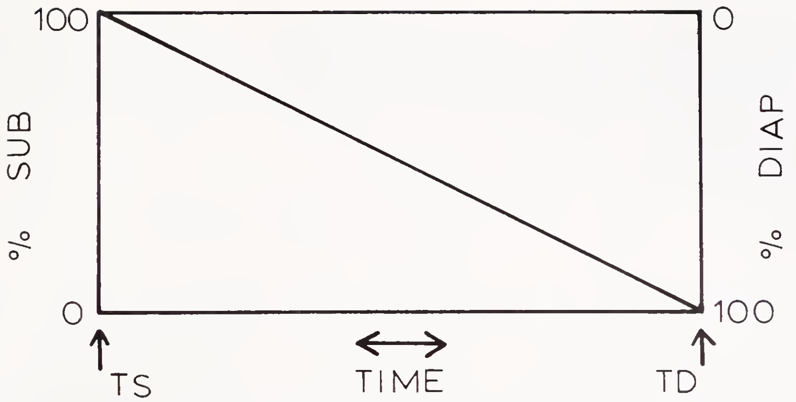

FIGURE 1. Hypothetical model of switch in egg production by individual females. At time$_{TS}$ the female is triggered to convert from 100% subitaneous to 100% diapause egg production. The actual time for conversion will depend on biological aspects of the female. Time$_{TD}$ indicates the switch-point for conversion in the reverse direction.

I suggest that each female in the population possesses a cumulative photoperiodic response threshold (see Fig. 1). For cumulative photophases (light period) greater than (or scotophases [dark period] less than) the threshold, only subitaneous eggs are produced. For cumulative photophases less than (or scotophases greater than) the threshold only diapause eggs are produced. The actual threshold may be considered as a window whose size is a function of the time required to completely convert from one egg type to the other, once the developmental pathway has been triggered. The duration of the transition phase may be affected by photoperiod, but it seems more likely that photoperiod initiates the switch and then the biochemical and physiological states of each female influence the time necessary to complete conversion. When heterogenous clutches are no longer produced conversion is complete. The light cue may induce changes in several parallel and/or consecutive pathways involving, for example, neurosecretions, hormone production, and other regulatory activities as is the case for insects (Beck, 1980). It is doubtful that such a conversion response could be instantaneous. As part of a previous unpublished study I collected eggs from individual field-collected females after 24 and 48 h in the laboratory. For some of these females heterogeneous clutches were obtained on both days. These observations support the suggestion that the transition phase is not instantaneous, but rather represents an interval during which time subitaneous and diapause eggs are produced. This type of reversible diapause response and transition period is comparable to that of the red spider mite *Metatetranychus ulmi* (Lees, 1953). That females can alter the type(s) of eggs produced in either direction is interesting from a physiological point of view. However, in the natural situation in Vineyard Sound the change should be unidirectional, from subitaneous to diapause egg production as daylength decreases in the fall. During the spring, daylength increases but the animals are not mature at the time when daylengths are short enough to induce diapause egg production (see Marcus, 1980, 1982). In Vineyard Sound diapause egg production should not precede subitaneous egg production, unless adult copepods were to survive the winter.

A problem confronting individuals that can switch egg production is when to change. For insect diapause, Taylor (1980a, b) suggests that if maximum fitness is measured as maximizing the number of offspring then individuals should produce subitaneous eggs for as long as there is sufficient time for those eggs to hatch and reach maturity. Beyond that point females should switch to diapause egg production, since the energy channeled into subitaneous egg production would be wasted. Therefore, selection should perpetuate an optimal threshold phenotype. This argument is applicable to the diapause response of *L. aestiva.* Although, some copepod females switch to diapause egg production early in September when daylength is greater than 12 h, others produce subitaneous eggs throughout the fall and never switch. Most females probably respond to intermediate values. The females that switch early maximize their contribution to the next season's population, whereas those that do not switch maximize their input to the current population and only make a genetic contribution to the following year's population by the diapause eggs of their subitaneous offspring.

Several factors may promote the heterogeneity of photoperiodic threshold phenotypes in Vineyard Sound. Although photoperiod is the primary environmental factor affecting the induction of diapause, other factors such as temperature and diet may modify its influence (Marcus, 1980, 1982). Unlike photoperiod, the timing and intensity of these parameters vary from year to year. Such fluctuations might lead to slight variations in the optimal phenotype each year. An additional source of variation would be gene flow with more southern populations. If these populations are adapted to their local environmental conditions, they might reflect different optimal threshold phenotypes due to latitudinal differences in the annual photoperiodic regimen. This type of interpopulation differentiation typifies insects that are capable of limited migration (Danilevsky, 1965). Thus if some migration was to occur between populations of *L. aestiva* this could promote heterogeneity. Elucidating the diapause response of populations from different latitudes should help clarify the population biology and evolution of this species.

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PHOTOPERIODIC AND TEMPERATURE REGULATION OF DIAPAUSE IN *LABIDOCERA AESTIVA* (COPEPODA: CALANOIDA)

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ABSTRACT

The calanoid copepod *Labidocera aestiva* was reared in the laboratory to ascertain the effects of photoperiod and temperature on subitaneous and diapause egg production. The results indicate that photoperiod is the primary stimulus regulating the type(s) of eggs produced by a female, but that the temperature regimen can modify this effect. Under short daylength photoperiods (*e.g.*, 8L:16D) subitaneous egg production is increased by increasing the temperature regimen from 15.0°C or 19.0°C to 25.0°C. However, under long daylength regimens (*e.g.*, 18L:6D) females appear to be unresponsive to temperature changes within the range of 15.0°C to 25.0°C. It is suggested that this response to photoperiod and temperature more accurately couples the life cycle of *L. aestiva* with seasonal environmental fluctuations.

INTRODUCTION

Research investigations of the last 10 years have made it increasingly evident that many marine calanoid copepods and other zooplankton have dormant stages (Onbe, 1978; Paranjape, 1980; Coull and Grant, 1981; review by Grice and Marcus, 1981). Diapause stages are a critical phase in the life cycle of these animals because they make possible long-term survival during periods unfavorable for continuous development. Elucidation of factors that influence the induction, maintenance, and termination of diapause should make possible more accurate predictions of seasonal fluctuations in the composition of plankton communities, and provide life history information needed to model and manage marine ecosystems.

At the northern edge of its range in Vineyard Sound, MA, *Labidocera aestiva* occurs seasonally in the plankton (Fish, 1925; Marcus, 1979). Adults appear in early summer and give rise to a series of overlapping generations. Nauplii, copepodites, and adults disappear from the plankton by the end of December, but the species remains in the area as a benthic diapause egg. The seasonal production of subitaneous and diapause eggs by *L. aestiva* in Vineyard Sound has been documented (Marcus, 1979), and it has been demonstrated for laboratory-reared females that photoperiod influences the type(s) of eggs produced (Marcus, 1980, 1982).

The results reported herein substantiate the importance of photoperiod as a factor regulating the phenology of *L. aestiva*. The data indicate that a short daylength photoperiod (*e.g.* 8L:16D) is the primary cue that triggers diapause egg production, but that the temperature regimen can modify this effect. The influence of these two environmental parameters on the life history pattern and population biology of *L. aestiva* in Vineyard Sound is discussed.

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MATERIALS AND METHODS

Labidocera aestiva individuals were reared from the first naupliar stage to reproductive maturity in 19 l glass carboys. The apparatus and procedure for rearing the nauplii, copepodites, and adults of *L. aestiva* is described in Marcus (1980). The same schedule for feeding, transfer of animals to clean seawater, and egg collection was followed throughout this study.

Experimental populations were initiated in each carboy with 200 nauplii derived from subitaneous or chilled diapause eggs. The eggs were produced by freshly collected females from Vineyard Sound or by females reared (first or second generation) in the laboratory. The animals in each carboy were reared at a constant temperature (14.5°, 18.0°, or 24.0° ± 1.0°C) and photoperiodic regimen (8L:16D, 12L:12D, or 18L:6D). Nine different combinations of photoperiod and temperature were analyzed. Seven were examined in this study, and two previously (Marcus, 1980). Two to five replicate experiments were conducted for each combination. When egg production began, the number of males and females in each carboy was equalized at 20–30 copepods of each sex. This was done by filtering the contents of the carboy according to the usual procedure (see Marcus, 1980) and removing adults at random from the filtrate with a wide-mouth pipette.

Eggs were collected from each carboy every 2–3 days for 6–14 days. For each collection a sample of 100–120 eggs from the total number of eggs obtained from the carboy that day, was placed in a dish containing glass fiber filtered seawater and incubated at 25.0°C and 12L:12D to hasten the time to hatching. After 4–5 days (initial) the number of hatched eggs was determined. The unhatched eggs were placed in jars containing filtered seawater and chilled at 5.0°C for a minimum of 40 days. At the end of this interval, the eggs were warmed at 25.0°C and the number that hatched (final) was ascertained. The proportion of subitaneous eggs produced was calculated by dividing the initial hatch by the total number of eggs in the sample. The proportion of diapause eggs produced was calculated by dividing the final hatch by the total number of eggs in the original sample. The remaining portion was classified as non-viable. All values were converted to percentages to facilitate comparison between samples and replicate carboys. Overall values for subitaneous, diapause, and non-viable egg production for each carboy were calculated as the mean of values for each collection day. After arc sine transformation of the percentage values, the analysis of variance was used to test for significant differences between means. Body size was estimated by preserving a minimum of 20 adult males and 20 females from each carboy in 5% buffered formalin, and subsequently measuring them to determine cephalothorax and total length.

RESULTS

The ages of laboratory-reared *Labidocera aestiva* at the onset of egg production were 26.6 ± 3.1 days at 13.5°–15.5°C, 21.5 ± 3.4 days at 17.0°–19.0°C, and 16.4 ± 2.4 days at 23.0°–25.0°C. More than 60% of the individuals survived to adulthood in each experimental carboy. The total number of eggs removed from a carboy was determined for most, but not all days that eggs were collected. Values ranged from several hundred to a few thousand eggs in each carboy per day. For some carboys the number of eggs collected per day varied by an order of magnitude. However, these differences had no obvious influence on the proportions of subitaneous, diapause, and non-viable eggs produced because the values were comparable in all instances.

The percentages of eggs that hatched initially at 25.0°C and that which hatched after prolonged exposure to 5.0°C are shown for each carboy in Tables I and II.

TABLE I

Percent hatch of eggs produced by females reared at 23.0°C–25.0°C under 8L:16D, 12L:12D, or 18L:6D. Eggs that did not hatch within 4–5 days at 25.0°C (initial) were incubated in jars at 5.0°C. The final hatch of these eggs at 25.0°C is indicated, as well as the cephalothorax (CT) and total body (TL) lengths in millimeters attained by adult males and females for each carboy. X = mean ± S.E. The blanks indicate that no eggs were incubated.

		Percent hatch (±S.D.)		Adult size attained (±S.D.)			
				Female		Male	
Photo-period	Carboy no.	Initial 25°C	Final 5°C	CT	TL	CT	TL
8L:16D	1	21.5 ± 25.2	87.5 ± 1.9	1.48 ± .06	1.98 ± .08	1.43 ± .04	1.89 ± .08
	2	37.5 ± 16.3	80.5 ± 13.5	1.48 ± .04	1.98 ± .05	1.41 ± .07	1.84 ± .06
	3	17.6 ± 12.0	82.3 ± 3.8	1.55 ± .05	2.06 ± .07	1.51 ± .05	1.96 ± .07
	4	51.8 ± 21.1	79.1 ± 2.8	1.46 ± .04	1.96 ± .06	1.40 ± .03	1.82 ± .05
X		32.1 ± 7.9	82.4 ± 1.8	1.49 ± .02	2.00 ± .02	1.44 ± .02	1.88 ± .03
12L:12D	5	81.0 ± 14.0	87.5 ± 8.5	1.59 ± .05	2.10 ± .06	1.43 ± .06	1.88 ± .05
	6	61.5 ± 29.0	45.0 ± 2.7	1.65 ± .05	2.20 ± .04	1.57 ± .01	2.10 ± .01
	7	65.5 ± 15.3	14.0 ± 14.0	1.59 ± .01	2.15 ± .04	1.53 ± .03	2.05 ± .01
	8	62.0 ± 20.4	18.0 ± 0.0	1.73 ± .07	2.32 ± .10	1.51 ± .07	2.07 ± .08
X		67.5 ± 1.6	41.1 ± 16.9	1.64 ± .03	2.19 ± .05	1.51 ± .03	2.03 ± .05
18L:6D	9	90.0 ± 3.2	2.0 ± 2.0	1.47 ± .05	1.95 ± .06	1.42 ± .05	1.85 ± .06
	10	84.3 ± 6.0	—	1.51 ± .07	2.01 ± .09	1.43 ± .07	1.86 ± .09
	11	88.7 ± 5.5	—	1.53 ± .10	2.03 ± .11	1.45 ± .05	1.94 ± .07
	12	91.8 ± 2.5	—	1.51 ± .08	1.99 ± .08	1.44 ± .05	1.89 ± .06
	13	91.5 ± 2.5	—	1.56 ± .06	2.08 ± .08	1.48 ± .05	1.98 ± .07
X		89.3 ± 1.4	2.0 ± 2.0	1.52 ± .01	2.01 ± .02	1.44 ± .01	1.90 ± .02

For each photoperiodic and temperature regimen the F values for the transformed data are less than the tabular values at the 5% level and therefore indicate no significant differences between the means of replicate carboys. The average percentages of subitaneous, diapause, and non-viable eggs produced at each photoperiodic and temperature combination are depicted in Figure 1. The values shown for 8L:16D and 18L:6D at 13.5°–15.5°C were derived from the results of Marcus (1980). The type(s) of eggs produced was affected primarily by photoperiod. Regardless of temperature, the greatest percentage of subitaneous eggs was produced at 18L:6D. Within the framework of a 24 h regimen, as the period of light was reduced (and dark increased) the percentage of subitaneous eggs declined, while the percentage of diapause eggs increased. For example, at 23.0°–25.0°C subitaneous egg production varied from 89.3% at 18L:6D to 67.5% at 12L:12D, to 32.1% at 8L:16D. Diapause egg production for these same conditions was 0.2%, 13.4%, and 55.9% respectively. The same trend in the types of eggs produced was observed for the experiments conducted at 17.0°–19.0°C, and at 13.5°–15.5°C (see Marcus, 1980). For all but one combination of photoperiod and temperature, 10.5–19.6% of the eggs were non-viable. The exception, 13.5°–15.5°C and 12L:12D, had a high proportion of non-viable eggs (35.1%) and an unexpectedly low percentage of diapause eggs (15.9%). However, the percentage of subitaneous eggs was not unusual.

The temperature regimen to which animals were exposed affected the types of eggs produced less than did photoperiod (Figure 1; Tables I, II). For the two cooler

Table II

Percent hatch of eggs produced by females reared at 17.0°–19.0°C under 8L:16D, or 12L:12D and 18L:6D and at 13.5°–15.5°C under 12L:12D. Refer to Table I for an explanation of the hatch and size data.

		Percent hatch (±S.D.)		Adult size attained (±S.D.)			
				Female		Male	
Photo-period	Carboy no.	Initial 25°C	Final 5°C	CT	TL	CT	TL
Temperature 17.0°–19.0°C							
8L:16D	14	14.4 ± 8.7	69.8 ± 5.8	1.60 ± .07	2.08 ± .31	1.50 ± .06	1.96 ± .08
	15	14.0 ± 5.6	83.7 ± 7.8	1.64 ± .09	2.17 ± .11	1.53 ± .07	1.97 ± .08
X		14.2 ± 0.2	76.7 ± 6.9	1.62 ± .02	2.13 ± .05	1.52 ± .02	1.97 ± .01
12L:12D	16	52.3 ± 25.1	79.0 ± 9.1	1.49 ± .06	1.97 ± .09	1.43 ± .06	1.88 ± .08
	17	47.3 ± 16.0	68.3 ± 2.7	1.57 ± .06	2.09 ± .08	1.46 ± .05	1.90 ± .07
X		49.8 ± 2.5	73.7 ± 5.3	1.53 ± .04	2.03 ± .06	1.45 ± .02	1.89 ± .01
18L:6D	18	90.3 ± 4.9	0.0	1.56 ± .06	2.05 ± .06	1.46 ± .07	1.90 ± .09
	19	86.0 ± 6.1	2.0 ± 2.0	1.53 ± .06	2.04 ± .08	1.47 ± .10	1.89 ± .08
X		88.2 ± 2.2	1.0 ± 1.0	1.55 ± .02	2.05 ± .01	1.47 ± .01	1.90 ± .01
Temperature 13.5°–15.5°C							
12L:12D	20	53.8 ± 19.4	18.5 ± 9.0	1.73 ± .09	2.34 ± .06	1.67 ± .07	2.34 ± .06
	21	56.5 ± 16.8	7.8 ± 10.7	1.72 ± .08	2.37 ± .12	1.62 ± .05	2.22 ± .08
	22	34.5 ± 17.1	14.0 ± 8.4	1.70 ± .08	2.32 ± .09	1.60 ± .04	2.13 ± .08
	23	51.3 ± 4.1	84.2 ± 6.3	1.64 ± .05	2.19 ± .07	1.57 ± .07	2.07 ± .11
X		49.0 ± 5.0	31.1 ± 17.8	1.70 ± .02	2.31 ± .04	1.62 ± .02	2.19 ± .06

regimens (13.5°–15.5°C, and 17.0°–19.0°C) the percentage of subitaneous eggs produced was comparable at each photoperiodic regimen, but at 23.0°–25.0°C the percentage of subitaneous eggs produced was markedly greater for the two shorter photoperiodic regimes (8L:16D and 12L:12D). At 18L:6D no temperature effect was observed. The percentage of diapause eggs obtained was minimal (0.2%) under 18L:6D at both 17.0°–19.0°C and 23.0°–25.0°C.

The body length of adult males and females reared in replicate carboys for each combination of photoperiod and temperature were similar (Tables I and II). For each experimental regimen females were longer than males (both cephalothorax and total length). Body length was inversely correlated with temperature. For the two cooler regimens body length and day length were inversely correlated, however, this relationship was not evident at 23.0°–25.0°C (Figure 2).

DISCUSSION

This study demonstrates the primary importance of photoperiod as a trigger for the induction of diapause in *Labidocera aestiva*, and a compensatory role for temperature. This is the first report of such an interaction for a marine copepod, although this relationship has been shown for many terrestrial and freshwater arthropods (Danilevsky, 1965; Hutchinson, 1967; Mansingh, 1971; Watson and

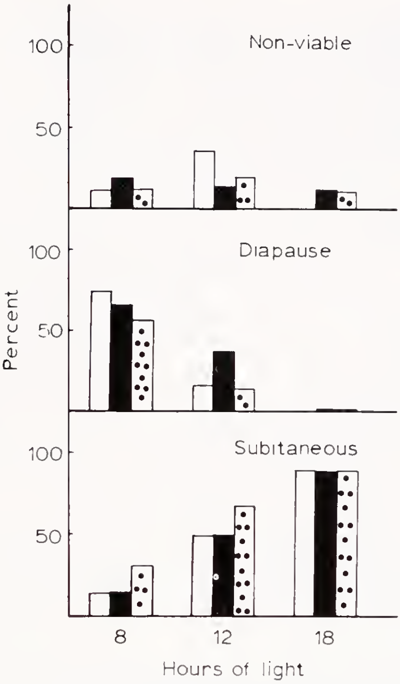

FIGURE 1. Subitaneous, diapause, and non-viable egg production (percent) by females reared under 8L:16D, 12L:12D, and 18L:6D at 13.5°–15.5°C (white), 17.0°–19.0°C (black), and 23.0°–25.0°C (dots). At 13.5°–15.5°C and 18L:6D only subitaneous egg production was determined.

Smallman, 1971; Beck, 1980). For each temperature regimen tested, the proportion of diapause eggs produced by the laboratory-reared *L. aestiva* was greatest at 8L:16D and declined as the period of experimental daylength was increased. This response was balanced by a reverse trend for subitaneous egg production. The percentages of subitaneous and diapause eggs produced was most affected by photoperiod, and the values are comparable to values reported for animals collected from Vineyard Sound at times of the year with correspondingly equivalent daylength periods (Marcus, 1979, 1980). Subitaneous egg production is maximal under long daylength conditions which prevail during the summer, whereas diapause egg production is greatest under periods of short daylength during the fall. Although photoperiod is the primary factor controlling diapause and subitaneous egg production, temperature may modify this influence. Within the range of temperature (15.0°–19.0°C) usually encountered in Vineyard Sound during September and October, the proportion of subitaneous eggs produced by laboratory-reared copepods is similar for any given photoperiod. However, for short daylength periods (*e.g.*, less than 12L:12D), warm temperatures (*e.g.*, 25.0°C) induce a greater proportion of subitaneous eggs (Table I; Figure 1). These photoperiods prevail in Vineyard Sound after mid-September until the vernal equinox. If water temperatures were unseasonably warm one fall, then the usual decline in subitaneous egg production should be postponed or more gradual. This would enable the population to take advantage of the longer growing season due to warmer temperature and maximize the number of individuals in that year's population. Although the effect of temperatures below 13.5°C were not investigated, if water temperatures were

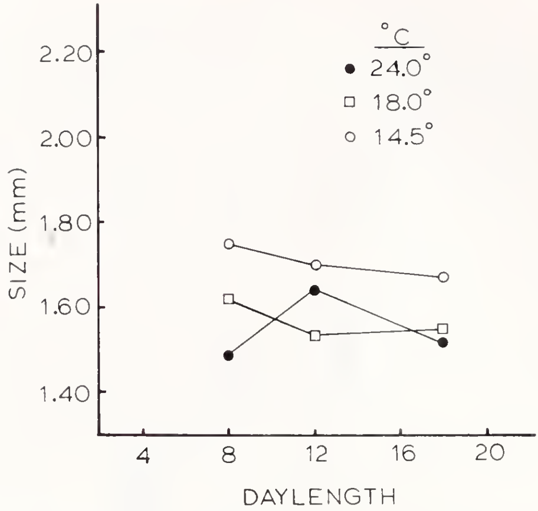

FIGURE 2. Cephalothorax length of adult females reared in the laboratory under different photoperiod and temperature regimens. The period of light is indicated. Temperatures are ±1.0°C.

unseasonably cold the reverse effect might occur, so that diapause egg production would increase earlier in the year. This response would ensure a maximum number of individuals in the next year's population. Adult *L. aestiva* disappear from the plankton by mid-December and do not appear again in Vineyard Sound until late May or June. At this time water temperatures are usually 15.0°–20.0°C and daylengths exceed 12 hours. Under long day photoperiods (*e.g.*, 18L:6D) in the laboratory, the type(s) of eggs produced is unaffected by temperatures of 15.0°–25.0°C. These results suggest that if water temperatures are unseasonably cool during the spring and early summer in Vineyard Sound, *L. aestiva* will nevertheless continue to maximize subitaneous egg production, and thereby promote rapid increase in the population size of the species during the summer. I have studied the population of *L. aestiva* in Vineyard Sound since 1977 and have never obtained diapause eggs from early summer females (Marcus, 1979; and unpublished observations). Thus under natural conditions the combined influence of photoperiod and temperature should more accurately couple the type(s) of eggs produced by *L. aestiva* with year to year environmental fluctuations.

The body lengths attained by males and females under the different temperature regimens are similar to the sizes of animals collected in Vineyard Sound (Marcus, 1979). The laboratory reared specimens also express an inverse relationship between body size and temperature as documented for *L. aestiva* from the field (Deevey, 1960; Marcus, 1979). Interestingly, for the two cooler regimens tested in the laboratory there also appears to be an inverse correlation between body length and daylength (Figure 2). This trend was noted previously by Marcus (1980). Two explanations suggested then to account for the observed relationship were that photoperiod influences body length indirectly either by affecting the thermal regime in the laboratory incubator, or the period of grazing activity and thus food consumption. These two hypotheses are applicable to the present investigation. A third possible mechanism is that photoperiod directly determines body size by affecting specific growth rate processes. Although temperature is probably the most important factor regulating body size of copepods (Coker, 1933; Aycock, 1942; Deevey, 1960), it has been shown for several insects (see review by Beck, 1980) and aquatic crustacea (Sandoz and Rogers, 1944; Auvray and Dussart, 1966; Starkweather, 1976; Dalley, 1980; Steele, 1981) that photoperiod directly influences some physiological, behavioral, developmental, and reproductive processes. Further experiments conducted under carefully controlled conditions specifically with regard to diet and temperature should help to clarify this intriguing and important problem.

The findings of the present and previous (Grice and Gibson, 1975; Marcus, 1979, 1980) investigations of *L. aestiva* suggest that the diapause response of marine copepods is analogous to the response of many terrestrial and freshwater arthropods (see reviews by Hutchinson, 1967; Elgmork and Nilssen, 1978; Beck, 1980). As in many of these organisms, the embryonic diapause of *L. aestiva* is triggered by a temperature compensated photoperiodic response. Other factors such as food quality or quantity, and the numerical density of *L. aestiva* may modify this induction response, but this has yet to be demonstrated for this species nor for any other marine crustacean. There are also similarities in regard to the maintenance and termination of diapause. The duration of the refractory phase (Watson and Smallman, 1971) is reduced by exposing diapause eggs to cold temperatures. Such treatment results in synchronous hatching once the eggs are subjected to warmer temperatures (Marcus, 1979). Like insects the life cycle stage that is dormant varies among copepods. Embryonic diapause is expressed by several calanoid copepods (see review by Grice and Marcus, 1981), and naupliar (Coull and Dudley, 1976), copepodite (Marshall and Orr, 1955; Davis, 1976) and adult (Coull and Grant, 1981) dormant stages are known for harpacticoid, cyclopoid, and other calanoid copepods.

From an evolutionary standpoint it is intriguing that a wide variety of organisms in the terrestrial, freshwater, and marine systems have evolved parallel adaptations and life history cycles to cope with environmental (biotic and abiotic) adversity. In some instances the pattern of variation of the selective factor is comparable (*e.g.* seasonal fluctuations of temperature), and the regulation of the response is similar (*e.g.* shortday photoperiodic response). However, not all environmental factors vary in the same way in each system. In terrestrial systems, small ponds, and the marine intertidal, water is a limiting factor, and during the dry periods many organisms undergo diapause. These stages are highly resistant to dessication. However, diapausing organisms inhabiting large lakes and the marine subtidal do not experience comparable periods of dryness. It would be valuable to determine whether the diapause stages of these animals are also resistant to desiccation, or whether they reflect some other attribute which is uniquely associated with their aquatic habit. Another parameter that differs among these habitats is the daily temperature regimen. In the terrestrial system many animals are exposed to a thermoperiod coincident with the day-night cycle. For some of these animals (see Beck, 1980) the temperature of the dark phase, but not the light phase, influences the diapause response. Such, daily temperature fluctuations are generally minimized in the ocean, and thus marine zooplankton may not reflect a parallel response. On the other hand, in thermally stratified waters zooplankton that migrate vertically might experience diurnal differences in temperature. If such animals moved into surface waters at night to feed and descended to deep waters during the day they would experience warmer temperatures at night than during the day. This daily thermoperiod would be the opposite of that encountered by animals in the terrestrial system and nighttime temperatures might influence the diapause response of such organisms in the reverse direction. Differences such as these might lead to subtle but distinct variations in the expression of diapause by organisms that dwell in the terrestrial, fresh water, and marine systems.

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CARBON AND NITROGEN FLUXES DURING DECOMPOSITION OF *SPARTINA ALTERNIFLORA* IN A FLOW-THROUGH PERCOLATOR[1]

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ABSTRACT

The carbon and nitrogen in *Spartina alterniflora* litter were monitored for 4 months during decomposition at 20°C in a flow-through percolator that simulates an aerobic, moist marsh. Both the evolution of CO_2 and the loss of carbon from the litter followed exponential decay kinetics (0.5% day^{-1} and 1.0% day^{-1}). At first both total organic carbon and total organic nitrogen were lost primarily in dissolved form at high rates, but this leaching ceased rapidly. The NH_4 added to the inflow was incorporated into the litter at about 0.25 mg $N \cdot g\ litter^{-1} \cdot day^{-1}$ initially, but the rate declined eventually to about 0.08 mg $N \cdot g\ litter^{-1} \cdot day^{-1}$ after 40 days. Nitrogen enrichment of the litter occurred in two phases with peaks on days 40 and 100. Nitrification started at day 30 and was the main consumer of NH_4. The final litter nitrogen concentration was 60% of the initial.

Microbially produced organic matter, calculated from incorporated NH_4, increased during decomposition to a constant value of 250 $mg \cdot g$ dry weight $^{-1}$ in the system; about 25% of the total dry weight of the litter. The efficiency of conversion of *Spartina* biomass to microbial organic matter was also biphasic and had maxima of 50% at the start and 70% at day 80 with an intermediate value of 20%. The continuous recovery of organic matter and CO_2 exported from the percolator allowed the microbial activity to be separated into different periods dominated by bacteria in the first 40 days and then by fungi for the remaining 80 days.

INTRODUCTION

In temperate zone saltmarshes, ninety-five percent of the annual aerial production (Mann, 1972) and a substantial amount (30–100%) of the below-ground production (Howarth and Teal, 1979; Hackney and de la Cruz, 1980) of *Spartina alterniflora* undergoes decomposition before it reaches higher trophic levels (for reviews, see Marinucci, in press; de la Cruz, 1973). The energy of production in a saltmarsh may be transferred to higher trophic levels either by consumption of the decomposed litter by marsh animals (Tenore and Hansen, 1980), or through reduction and subsequent re-oxidation of sulfate by chemosynthetic bacteria (Peterson *et al.*, 1980).

Of the several ways to study overall decomposition *in situ*, litter bags are used most often. With this technique one can follow net changes in chemical and microbiological composition of the litter, but can not separate the gross chemical

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exchanges between water and litter or air and litter. Furthermore litter bag data are highly variable because of poor control of such things as oxygen tension, humidity, temperature, and nutrient availability in field experiments. Another *in situ* method used flow-through chambers to study the effect of water quality on *Spartina* decomposition (Lee *et al.*, in press). In these chambers the microbial and meiofaunal populations were altered from their natural state to measure the relative impact on decomposition of these two decomposer communities. The system does require extensive field maintenance and still has some of the drawbacks of integration of a number of environmental variables.

Another way to study decomposition is to simulate the natural environment in the laboratory (Burkholder and Bornside, 1957; Gosselink and Kirby, 1974; Haines and Hansen, 1979). In such experiments, *Spartina* litter decomposes in water in shaken flasks. These flask experiments, however, do not recreate the highly moist but aerobic conditions found in the litter layer of the marsh, and they allow buildup of metabolic products which can inhibit decomposition. In practice, flask studies have been run for only short periods; they have had a maximum duration of 40 days and most did not exceed 2 weeks.

The environment of a marsh surface can also be simulated in a percolator. This device rapidly recirculates water through a column of soil or particles suspended above a water reservoir (Lees, 1949; Temple, 1951); however, the water in the percolator is not renewed, and eventually microbial processes become inhibited because of metabolite buildup.

In this study I modified a percolator design to simulate the environment of decomposition found in the low marsh. In this new design, the water is continually replenished and metabolic products do not build up. In addition the apparatus allowed adequate control over the various environmental components that affect aerobic decomposition with the result that the variability in the data was greatly reduced. Furthermore, I could account for all major exchanges of carbon and nitrogen between water and litter or air and litter. Other advantages of this percolator are a large sample capacity of 100–125 cm^3 (6–10 g dry weight of *Spartina* litter), an incubation of the litter in a non-submerged but moist environment, and separately controlled percolation and water flow-through rates. More pragmatic features of this design are easy disassembly, chemical inertness due to the all-glass construction, and a relatively low cost. Finally, with this design I could follow decomposition for many months.

Results of *in situ* decomposition studies show that carbon and weight are lost exponentially from litter. Nitrogen, however, is first rapidly lost through leaching from litter in the marsh, then re-incorporated from the environment, and finally lost from the litter at a slow exponential rate (Odum *et al.*, 1973). During this latter stage, meiofauna increase the mineralization of *Spartina* litter to CO_2 (Lee *et al.*, in press). Because of the steady loss of carbon and increases in nitrogen during decomposition, there is a gradual decrease in the carbon:nitrogen ratio. The nitrogen increases are usually attributed to microbial activity in the detritus but little is known of the populations or processes involved. Microscopic examination of *Spartina* litter showed that bacterial biomass is relatively small in aged detritus (Odum *et al.*, 1979) while the types and concentrations of sterols suggest that fungal biomass may predominate in this litter (Lee *et al.*, 1980). Other biochemical measurements on leaf litter in a Florida estuary suggest that the initial bacterial colonization of litter was followed by fungal growth (Morrison *et al.*, 1977). Additional evidence for the importance of fungi comes from the high amounts of non-protein nitrogen in detritus; it is as high as 30% of the total litter nitrogen by dry weight (de la Cruz and Poe, 1975, Odum *et al.*, 1979). Between 25 and 50% of this non-protein fraction of nitrogen is likely chitin, which can originate from fungal cell walls (Odum *et al.*, 1979). However, this apparent fungal dominance in the leaf litter was reduced when the litter was grazed by small crustaceans (Morrison and White, 1980).

Nitrogen incorporation from the surrounding media into *Spartina* litter had also been noted in laboratory experiments (Gosselink and Kirby, 1974) and the presence of dissolved inorganic nitrogen in the media increased decomposition rates (Haines and Hansen, 1979). Other workers show that in the laboratory the presence of meiofauna increased CO_2 evolution in the latter stages of the process (Lee *et al.*, 1976); this corroborates the results found in the field (Lee *et al.*, in press). Bacterial and overall microbial growth efficiencies, calculated from both the nitrogen and the ATP content of litter, were between 20 and 60% (weight of calculated microbial biomass produced per dry weight of plant litter decomposed) (Gosselink and Kirby, 1974; Burkholder and Bornside, 1957; Fallon and Pfaender, 1976; and Haines and Hansen, 1979). The efficiency of fungal growth, however, was as high as 82% on a substrate of *Spartina* leachate (Fallon and Pfaender, 1976). These efficiencies of bacterial growth are within values (40–60%) observed for both pure and mixed growth of bacteria on glucose in minimal medium (Payne, 1970). Fungal growth efficiencies on optimal medium (65%) are higher than bacterial values (Payne, 1970), but did not approach the high value reported by Fallon and Pfaender (1976).

The present study was carried out to develop and test the use of flow-through percolators for the study of the chemical and biological processes and controls during *Spartina* decomposition. In one test I compared the time course of decomposition of *Spartina alterniflora* in litter bags in a natural salt marsh with decomposition of the same litter in percolation chambers. In another test, I compared decomposition in seven percolators and computed standard deviations of measurements of weight, carbon, and nitrogen in the litter, the amount of carbon and nitrogen exported from the percolators in the water, and the respiration as calculated from loss of CO_2 in the air stream. Finally, a budget of carbon and nitrogen entering and leaving the percolators over a 4 month period was constructed both to test the accuracy of measurements and to investigate the relative importance of various processes.

MATERIALS AND METHODS

Laboratory experiment and apparatus

The litter used in each flow-through percolator was 6 g dry weight (5.1 g ash free dry weight) of air dried, mechanically shredded (commercial garden shredder) *Spartina alterniflora* leaves and stalks (approximately 10–20 × 1–2 mm after shredding) harvested in August from green plants from an undisturbed saltmarsh (Island Beach, N. J.). A gram of dry weight of litter was 13% ash and the ash free dry weight contained 48% C and 1.28% N.

In the percolators (Fig. 1), the litter sample was held in the column (A) above the water level by glass nubs and a small amount of coarse glass wool. An air lift pump with a flow of 100 ml/min (D) rapidly recycled water from the reservoir (B) to C and then A. The water level was maintained below the litter by the position of the air lift junction, which is just below the bottom of the litter sample. Input water was added (E) to the percolator with an Ismatic® peristaltic pump (flow rates between 0.32 and 0.42 ml/min). Residence time of the 200 ml of water in

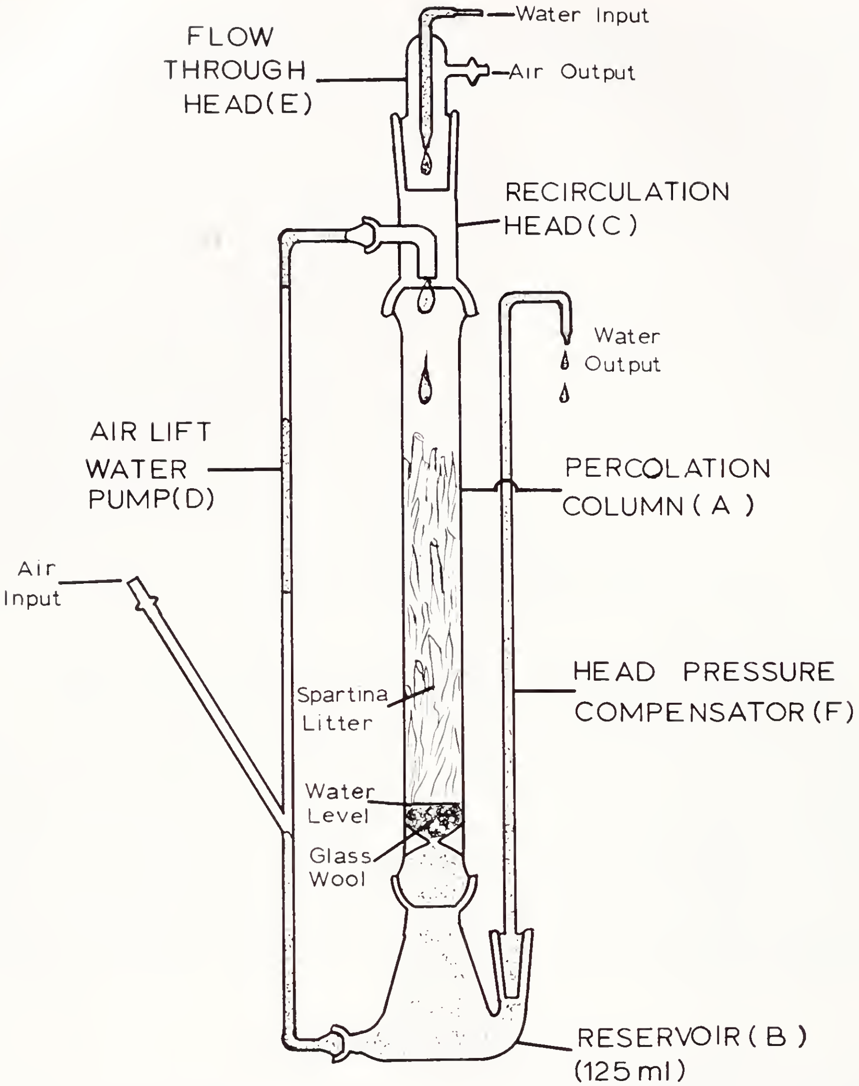
Water Input
FLOW THROUGH HEAD(E)
Air Output
RECIRCULATION HEAD(C)
Water Output
AIR LIFT WATER PUMP(D)
PERCOLATION COLUMN (A)
Air Input
HEAD PRESSURE COMPENSATOR (F)
Spartina Litter
Water Level
Glass Wool
RESERVOIR (B) (125 ml)

FIGURE 1. Flow-through litter percolator. Unit is approximately to scale with overall length of 58 cm (Construction details in Marinucci, 1981, or from author; modified units commercially available from Belco Glass Co., Vineland, NJ, Cat Nos. 9105-S0009 and 9105-S0011). Assembly of unit requires two #12 and two #35 ball and socket clamps (Arthur H. Thomas Co., Philadelphia). Joints should be either coated with a small amount of silicone grease or lined with teflon (Ace Glass Co., Vineland, NJ) to prevent small leaks.

the system was 6 to 8 hrs. The water leaves the system through the head pressure compensator (F) which was high enough to balance the head pressure generated by bubbling air through a total of 30 cm of KOH solution in the two CO_2 traps connected in series to the air output (E) (Fig. 2). Each trap was a 25 × 250 mm test tube with 50 ml of 0.2 *N* KOH. Influent air was bubbled through an alkali solution (15% KOH) and then distilled water to remove CO_2 and replace moisture in the air stream. The input was artificial sea water (Utility Marine Mix, Chemical

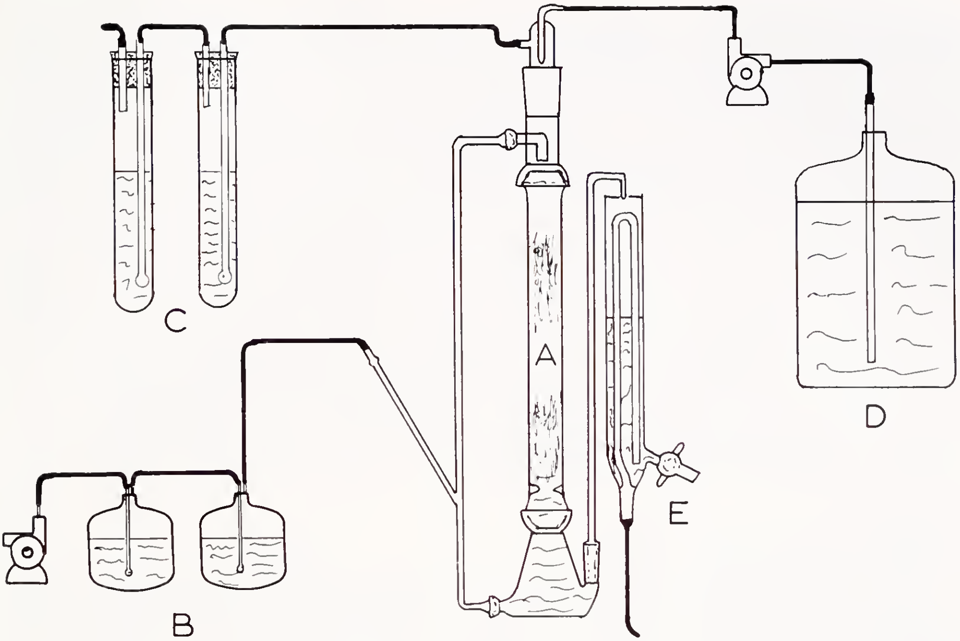

FIGURE 2. Diagram of the system showing the percolator (A), the influent air scrubbers (B), the effluent scrubbers (C), the reservoir (D), and the self-emptying collection device (E).

Co., Paterson, N. J.), diluted to 22‰ salinity and containing 9 mg $N \cdot liter^{-1}$ as $(NH_4)_2SO_4$. All percolators were operated at 20°C in an incubator and were kept in total darkness to prevent algal growth. One percolator was poisoned with 0.1% sodium azide in the inflow water to test for abiotic changes in the litter.

Comparative in situ *study*

To compare the percolator experiments with *in situ* experiments, I also measured decomposition in a New Jersey salt marsh with the same litter as used in the percolators (for details see Marinucci and Bartha, in press). The initial chemical composition of this litter was the same as that in the percolator experiments. In these experiments, which began in early to mid-summer, litter was placed in both high and low marsh locations in 18 × 30 cm litter bags with 2 mm mesh. Periodically, a bag was removed from the marsh and the contents analyzed for total dry weight, ash free dry weight, total carbon, and total nitrogen.

Chemical analysis of nitrogen and carbon components

Evolved CO_2 was trapped and measured by titration of the residual alkalinity after precipitation of carbonate with excess $BaCl_2$ (5 ml of 1 *M* solution). The chemical equivalents of CO_2 as carbonate in each trap was equal to ½ the equivalents of base neutralized. Ammonia was analyzed immediately after sampling in both inflow and outflow waters with an ammonia gas sensing electrode and standard addition techniques (Orion Research Inc., 1975; Gilbert and Clay, 1973; Srna *et al.*, 1973). Samples of influent and outflow water were periodically collected and frozen in polyethylene bottles for nitrate, nitrite, total organic nitrogen, and total organic carbon analyses. Nitrate was determined with the brucine-sulfate procedure (Standard Methods, 1971, Part 213 C). Nitrite was measured with the sulfanilic acid technique (Taras, 1971, Part 134). Total organic dissolved and suspended nitrogen (TON) was analyzed as ammonia with a gas sensing electrode after Kjeldahl digestion (Mertens *et al.*, 1975). Total organic dissolved and suspended carbon (TOC) was measured with a Beckman organic carbon analyzer after samples were acidified and scrubbed with nitrogen for removal of carbonates. Initial and final nitrogen, carbon, and ash-free dry weight measurements were made on representative subsamples of the fresh and decomposed litter from all percolators. Procedures used for the litter were similar to those outlined for analysis of field litter samples (Marinucci and Bartha, in press). Briefly, nitrogen was determined by electrode measurement of dissolved ammonia gas in alkali-adjusted and diluted Kjeldahl digests. Carbon content of the litter was gravimetrically measured as carbonate after wet oxidation of the carbon and subsequent absorption of CO_2 on Ascarite®. The ash-free dry weight of the litter was calculated from net weight changes in samples dried to constant weight and then combusted at 550°C for a minimum of 2 h.

Calculation of chemical changes

Total material either accumulated in the litter or removed in the outflow water was calculated as follows. Uptake rates were the product of the mean difference in concentration between input and output water and the mean flow rate for the time interval. The total material was the summation of the products of the uptake rates and the time interval between the samples.

Values for days in which no samples were taken were calculated from linear interpolation of the adjacent data. The carbon and nitrogen contents of the litter, as well as incorporated nitrogen, were then calculated for each day of the experiment with equations 1, 2, and 3:

$$\text{Total litter carbon} = \text{Initial carbon} - \text{Carbon dioxide} - \text{TOC} \qquad (1)$$

$$\text{Total litter nitrogen}$$
$$= \text{Initial nitrogen} + \text{Ammonia utilized} - \text{TON} - \text{Nitrate} - \text{Nitrite} \qquad (2)$$

$$\text{Ammonia incorporated into litter} = \text{Ammonia utilized} - \text{Nitrate} - \text{Nitrite} \qquad (3)$$

Data shown in this paper are the mean results of seven identical percolator experiments run at two different times.

RESULTS AND DISCUSSION

Comparison of percolator decomposition with in situ *litter bag results*

Decomposition of litter was similar in the percolator and in bags from the high and low marsh (Fig. 3). Data points (n = 3) from these litter bag experiments had a standard deviation of around ±5–8% while percolator data points had an average deviation of around ±1–2%. Decomposition rate constants were calculated for these experiments by regression of the natural logarithm of the total litter carbon against time (Table I). The percolator data correlated best with this exponential function and falls between the decay rates for litter in the high and low marsh. Carbon loss rates of 0.86 to 2.31%$\cdot$day^{-1}, which were determined by Lee *et al.* (in press) from $^{14}CO_2$ evolution from radiolabeled *Spartina* in a field microcosm in the low marsh also bracket the percolator decay rate.

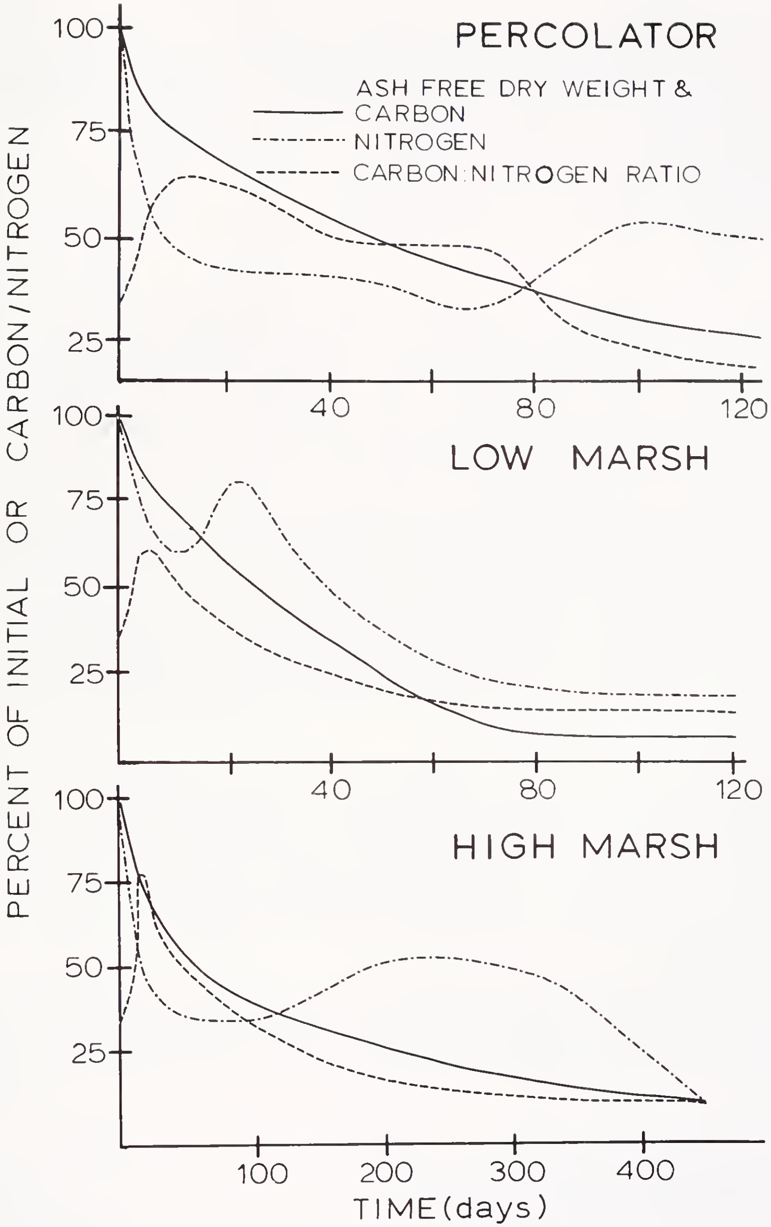

FIGURE 3. Decomposition of *Spartina alterniflora* litter in percolators and in litter bags in the high and low salt-marsh. Carbon and nitrogen quantities are expressed as percent of the initial amount while the C/N ratios are actual values. Field data were redrawn from Marinucci and Bartha (in press).

Nitrogen changes were much more complex in percolator-decomposed litter than in the litter bag study. However, there was a general pattern of net nitrogen accumulation in the litter after an initial rapid loss (see also Mann, 1972; Odum *et al.*, 1973). Overall, the C:N ratios increase sharply from the initial level of 38 but all eventually decline to the relatively nitrogen-rich level of 18 to 20. However,

TABLE I

Decay rates in litter bags and in percolator.

	Rate (% per day)	Calculated half-life (days)	R^2
Percolator	0.98 ± 0.03	70	0.99
Litter Bags High Marsh	0.44 ± 0.05	158	0.80
Litter Bag Low Marsh	2.20 ± 0.20	31	0.81

in the percolator both C:N ratio and absolute nitrogen have a bimodal curve which is not normally observed in litter bag experiments. I believe this curve is real and was found because of the high frequency of sampling and low variability in samples in the percolator when compared to field studies (2–3 days versus 1–2 weeks).

The slower rate in the percolator than in the low marsh can be attributed to several factors. The incubation temperature of the percolator was 20° C, which is somewhat lower than the average summertime marsh temperatures of 22 to 28°C. The percolator also excluded small crustacean and molluscan grazers which are present in the litter bags and which can increase the decomposition rate (Morrison and White, 1980). Meiofauna, which were not monitored in my study may have been absent and therefore decreased overall decomposition (Lee *et al.*, 1976). Finally, though the percolator environment is continually moist, the tidal currents were absent from the percolator. These currents can mechanically fragment litter and result in an increase of the decomposition rate (Gosselink and Kiby, 1974).

Carbon and nitrogen budgets

Both the carbon and nitrogen budgets for the percolators balanced quite well as 99% of the carbon input and 107% of the nitrogen input was accounted for (Tables II and III). The majority of carbon lost after 120 days (approximately 50%) was as CO_2 and only a quarter of the initial carbon remained as litter carbon (Table II). Another quarter of the carbon was lost as dissolved and suspended total organic carbon (TOC) in the outflow water. This includes a very small amount of particulate matter.

Of the total nitrogen in the decomposition process (initial and added ammonia nitrogen), about 39% was either retained or immobilized by the detritus at the end of the experiment (Table III). The majority (61%) of total nitrogen processed by

TABLE II

Total carbon inputs and outputs during aerobic decomposition of Spartina alterniflora *litter in a flow-through percolator at 20°C for four months.*

	Weight (mg)	Mean % of total and initial
Input:		
Initial Litter Carbon	2462 ± 15	—
Output:		
Final Litter Carbon	655 ± 49	26
Carbon Dioxide Evolved	1095 ± 32	45
Carbon (TOC)	694 ± 37	28
Total Carbon Output	2445 ± 77	—

TABLE III

Total nitrogen inputs and outputs during aerobic decomposition of Spartina alterniflora *litter in a flow-through percolator at 20°C for four months.*

	Weight (mg)	Mean % of total input nitrogen	Mean % of initial litter nitrogen
Input:			
Initial Litter Nitrogen	63 ± 0.4	39	100
Ammonia Utilized	98 ± 4.5	61	156
Total Nitrogen Input	161 ± 4.2	—	256
Output:			
Final Litter Nitrogen	43 ± 2.8	25	68
Nitrate Produced	52 ± 5.2	29	82
Nitrite Produced	2 ± 0.2	1	2
Nitrogen (TON)	74 ± 7.8	43	117
Total Nitrogen Output	173 ± 10.9	—	275

the decomposing litter was from external sources (ammonia-N). A significant quantity of ammonia-nitrogen was converted to nitrite and nitrate through microbial nitrification processes. The largest quantity of nitrogen exported, however, was in the form of either dissolved organics, suspended micro-particulates, or microbial biomass; this was 43% of the total output nitrogen (TON). The large fraction of the nitrogen that appeared as TON could be attributed to the leaching of nonsenescent grass which I used as my source of litter. The TOC may likewise have been affected by this type of litter.

Gross changes in chemical components

About 20 to 25% of the carbon was leached as dissolved carbon in the initial stages of the decomposition (Fig. 4). Afterwards, carbon was lost at a steady rate

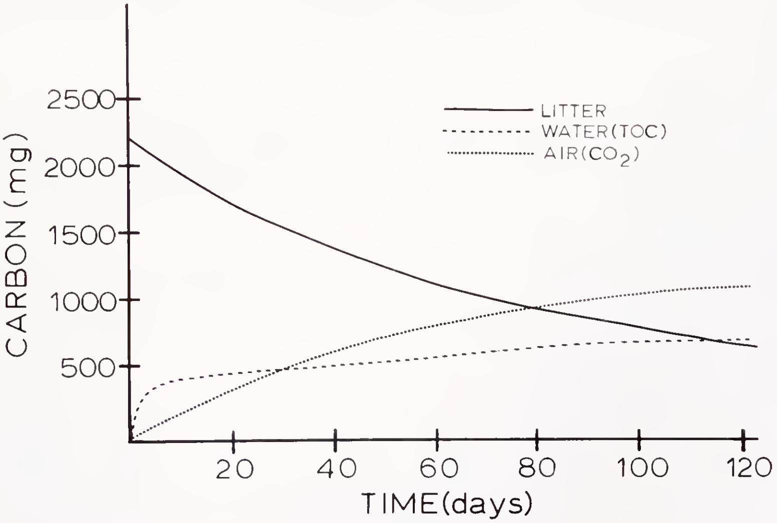

FIGURE 4. Cumulative amounts of carbon gained or lost by *Spartina alterniflora* litter during aerobic decomposition in a percolator at 20°C.

of about 2.5 mg C · day^{-1}. The CO_2 evolution was initially less rapid than the total organic carbon (TOC) losses but was much more constant during the experiment. The rate of CO_2 evolution did, however, decrease as litter was consumed.

The rate of CO_2 evolution from each percolator decreased over time, and the total evolved approached a value of 1250 mg C (Fig. 4). This value and the 700 mg TOC substracted from the initial carbon (2450 mg) leaves 500 mg of undecomposable carbon. This is probably the lignin fraction which remains fairly intact after cellulose mineralization (see Swift *et al.*, 1979, Chapter 2). This amount of recalcitrant carbon, about 20% of the initial carbon, is the same as the lignin found in fresh *Spartina alterniflora* by MacCubbin and Hodson (1980).

Nitrogen losses were also rapid at the start of decomposition with 66% of the initial nitrogen lost as TON in the first 10 days (Fig. 5). The TON was continually lost for the remainder of the experiment, and the TON loss rate decreased to a constant value of 0.5 mg N · day^{-1} after day 30. Cumulative TON lost during decomposition surpassed by a small quantity the initial nitrogen of the *Spartina* litter. In litter poisoned with sodium azide (data not shown), there was a TON total loss of only 50%, 9/10ths of which was lost in 20 days. This indicates that a lot of the TON loss from the litter was microbially mediated.

The ammonia utilization curve shows net nitrogen incorporation from the water into the decomposing litter (Fig. 5). This incorporation was initially rapid and constant (0.25 mg N · g litter^{-1} · day^{-1} as NH_4), but became slow and variable after 40 days (approximately 0.08 mg N · g litter^{-1} · day^{-1}). Then ammonia oxidation to nitrate and nitrite became important. This process of nitrification was first detected after day 30 of the experiment, and significant quantities of products were measured after day 40. The slow development of nitrification in the percolation system may have been the result of the relatively slow generation time of the nitrifiers of 20–40 hrs (Focht and Verstraete, 1977). Nitrification may also have been suppressed by the high dissolved organic matter in the percolators before day 30; this is significantly reduced about the same time as commencement of nitrification (data not shown).

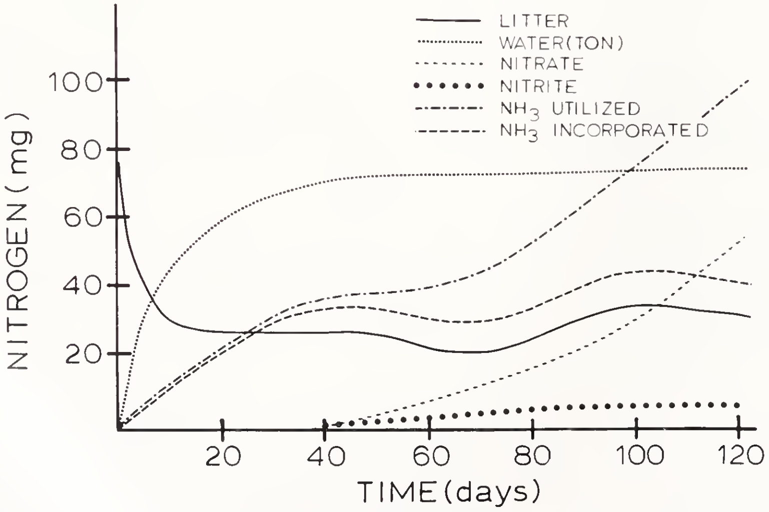

FIGURE 5. Cumulative amounts of nitrogen gained or lost by *Spartina alterniflora* litter during aerobic decomposition in a percolator at 20°C.

The high amounts of TOC and TON produced during the first few days of the study probably result from the type of litter used in this study. This grass was actively growing; therefore, much of the nutrients were still in the stems and leaves. Scenescent grass, which is the major litter input in nature, would have lost most of these nutrients to the roots for storage.

The soluble organic matter lost after the initial rapid leaching of litter at the beginning of the experiment (TOC and TON after day 30) had a mean C/N value of 5. Pure protein and micro-organisms can also have this low C/N value. However, since the outflow water was nearly devoid of culturable microorganisms (J. C. Hunter, personal communication), this organic material was probably either soluble protein, amino acids or polypeptides.

The percolator which was poisoned as a control for abiotic litter decay did not evolve any CO_2, nitrate, or nitrite (data not shown) and did not accumulate any nitrogen. About 20–25% of the carbon as TOC and 45–50% of the nitrogen as TON was leached abiotically during the first few days of the study, but this loss then ceased and the litter did not decompose further.

Representative values of rates and concentrations are given in Table IV to illustrate the levels found.

Contribution of microbes to the litter

Since a large percentage of the initial nitrogen is rapidly leached from the litter in the early stages of decomposition, I assumed that the incorporation of ammonia nitrogen into the litter is an indicator of the magnitude of microbially produced organic matter. This assumption was supported by the fact that ammonia was accumulated in litter at the same time as TON leached from the litter (see Fig. 5). However, it is not known whether this incorporated nitrogen remains in microbial biomass (bacteria, hyphae) or becomes a part of microbial slimes or fragments of cell walls and hyphae. For the purpose of this paper I assume that the composition of the microbially produced material is the same as the composition of microbes. Accordingly, the incorporated ammonia nitrogen was divided by 0.13% to calculate the total mass of the microbially produced organic matter (Gosselink and Kirby, 1974). This conversion factor is near the upper range of values of

TABLE IV

Approximate rates of inputs, uptakes, and outputs; concentrations of outputs; and variation of cumulative data for the chemical components measured during aerobic decomposition of Spartina alterniflora *in a percolator for 4 months at 20°C.*

Chemical component	Inputs ($mg \cdot day^{-1}$)	Uptake ($mg \cdot day^{-1}$)	Output ($mg \cdot day^{-1}$)	Median output concentrations ($mg \cdot l^{-1}$)
CO_2-C	0	0	5–18	—
Carbon (TOC)	0	0	0–7[1]	6.30
NH_4-N	4–5[2]	0.3–2.3	2.7–4.7	5.40
NO_3-N	0	0	0.02–1.20[3]	1.00
NO_2-N	0	0	0.01–0.10[3]	0.09
Nitrogen (TON)	0	0	0.02–1.4[4]	1.26

[1] TOC lost by leaching in the first 5 days was approximately 250 mg $C \cdot day^{-1}$.

[2] Input NH_4-N concentration was approximately 9.0 mg $N \cdot l^{-1}$.

[3] Nitrification products were first detected at day 30.

[4] TON lost by leaching was approximately 10 mg $N \cdot day^{-1}$.

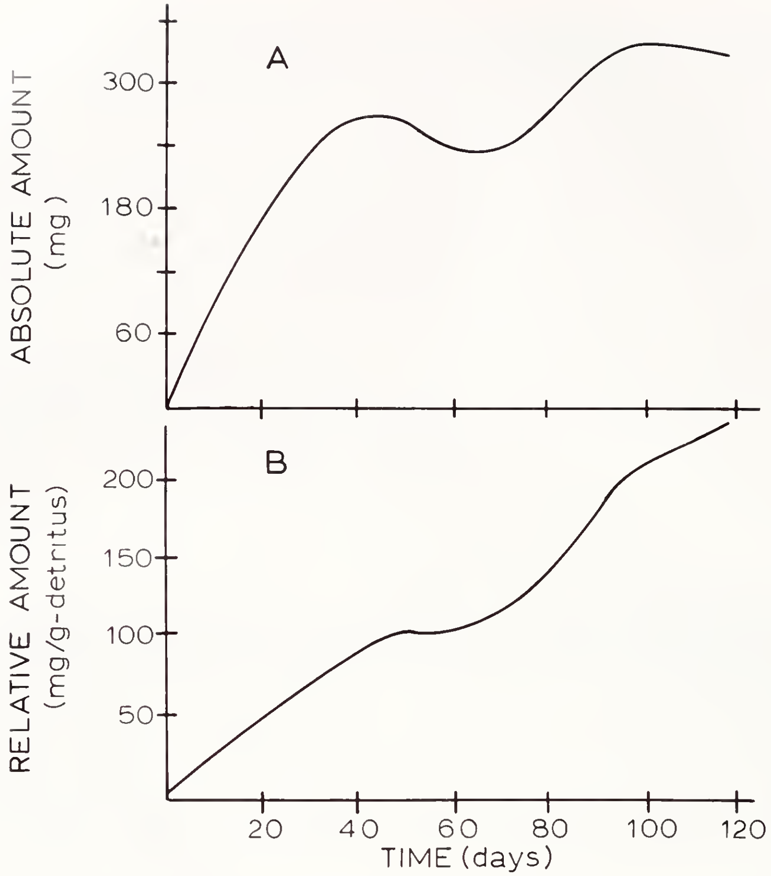

FIGURE 6. Absolute (A) and relative (B) amounts of microbially produced organic matter in decomposing *Spartina alterniflora* litter in a percolator at 20°C.

microbial nitrogen content (microbes are 5.2 to 13.6% N) and thus makes the most conservative estimate of microbial produced organic matter (Lipinsky and Litchfield, 1970). Absolute amounts of the microbially produced organic matter increased rapidly in the first few days of decomposition and then became relatively constant, though there was a slight biomodal shape to the curve (Fig. 6). The amounts per gram of litter, on the other hand, increased in a nearly linear fashion throughout the experiment. The calculated microbial production in the resultant litter was 250 mg $\cdot$ g^{-1} or 25% by dry weight of detritus at the end of the experiment.

The bimodal shape of both the C/N curve (Fig. 1) and the absolute amount curve (Fig. 6A) indicates a two-phased microbial enrichment of the litter which is also seen in decomposition data for other types of macrophyte litter in aquatic systems (Hunter, 1976; Andersen, 1978; Seki and Yokohama, 1978; Blackburn and Petr, 1979; Kruczynski *et al.*, 1979) and in terrestrial systems (Howard and Howard, 1974). However, this two-phased pattern was not very pronounced in these cited works, and therefore was not discussed by these authors.

The initial nitrogen enrichment during decomposition in the first 40 days was probably the result of rapid colonization by heterotrophic microbiota which exploited the more easily decomposable fractions of the litter. The second step of nitrogen build-up in the remaining 80 days could indicate a shift of the microbiota in response to a depletion of the more readily utilizable substrates. This in turn causes a buildup of a population which attacks the less available bio-polymers (see Swift *et al.*, 1979, Section 4.5). In *Spartina* litter, Lee *et al.* (1980) found a similar pattern for fungal biomass calculated from the ergosterol content but only a one step increase of bacterial biomass in the same litter (direct counts). Since the more recalcitrant lignocellulosic components of the litter are degraded primarily by fungi, it is likely that a shift from primarily cellulolytic fungal population to lignolytic types was responsible for the two step enrichment. Extrapolation of lignin mineralization data gathered *in vitro* strongly indicates that most (approx. 70%) of the lignin fraction of *Spartina alterniflora* is not decomposed by the microbial community present during the initial stages of decomposition (MacCubbin and Hodson, 1980). Further decomposition of this litter requires a subsequent increase in the population of a lignolytic microbiota.

The second step of nitrogen enrichment of the litter correlated with the increase in nitrate and nitrite in the outflow waters (Fig. 5) so it is possible that nitrifying bacteria contributed to the organic matter. To test this, the maximum contribution of chemoautotrophic microbial biomass to the litter was calculated from the amount of ammonia oxidized to nitrite and then to nitrate. This value was derived from the nitrate production data and from published conversion factors (Focht and Verstraete, 1977) and is about 10^5 cells · mg NO_3-N^{-1}. I assumed some diameters and densities for small rod-shaped nitrifying bacteria (Ferguson and Rublee, 1976), and then calculated a mean dry weight for the nitrifier cell of 3.05×10^{-14}g. With these two factors and the total nitrate production at the end of the study (Table III), only a maximum of 0.2 mg of nitrifier biomass could be produced in this experiment. If the maximum rate of nitrate production (Table IV) as an indicator of active nitrifier biomass (10 g NH_3-N was oxidized to $NO_3 \cdot day^{-1} \cdot g\ cell^{-1}$), it can be calculated that only about 0.12 mg of nitrifiers were active at the end of

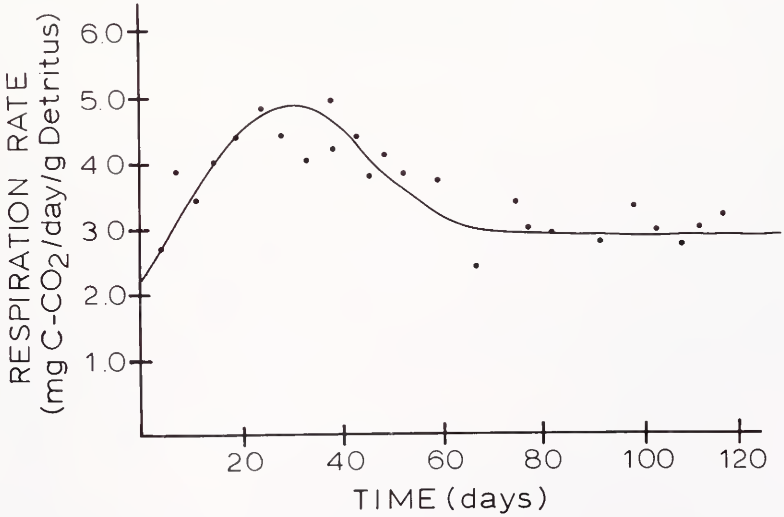

FIGURE 7. The CO_2-C evolution rate from *Spartina alterniflora* litter which was decomposed in percolator at 20°C. The data were corrected for litter weight. Total mass of litter was calculated from litter carbon changes (0.48 g C · g $AFDW^{-1}$).

the experiment. Both of these values are insignificant when compared to 320 mg of microbially produced organic matter in the litter at the end of the experiment.

The respiration rate of the litter increased continually from about 2 mg C$\cdot$day$^{-1}\cdot$g detritus^{-1} at the start of the experiment to about 5 mg C$\cdot$day$^{-1}\cdot$g detritus^{-1} at day 25 (Fig. 7); this agrees with the rapid increase in the microbially produced organic matter (Fig. 6). Afterwards, respiration decreased to a relatively steady level of 3.5 mg C$\cdot$day$^{-1}\cdot$g detritus^{-1} for the duration of the experiment. Such a decrease in respiration rate without a simultaneous decrease in the relative amounts of microbially produced organic matter strongly supported the hypothesis that the majority of this organic matter is not active microbial biomass (Lee *et al.*, 1980). The same pattern was also seen in O_2 respiration in field decomposed *Spartina alterniflora* (Lee *et al.*, 1980). They attributed the pattern to environmental temperature changes, but because a similar pattern was found in the percolators at a constant temperature, their conclusion may not be correct.

Instantaneous conversion efficiencies of plant biomass to microbially produced organic matter (Fig. 8) were calculated from NH_3-N litter uptake data (Fig. 5) and CO_2-C evolution data (Fig. 4). The curve accentuates the bimodal nitrogen enrichment of the litter, and also suggests a two-phase microbial colonization of the litter by microbiota. Peak conversion efficiencies averaged around 60–70%, though individual points were as high as 90%. Though these extremely high values might be artifacts of the iterative nature of the efficiency calculation or of the value of nitrogen content of the microbiota, the trends in microbial growth dynamics are definitely present. Furthermore, the mean peak efficiencies are reasonable and consistent with those found in pure culture experiments for both bacteria (40 to 60%) and fungi (65%) (Payne, 1970). The initial microbial conversion efficiency could be associated primarily with bacterial growth as suggested by Morrison and White (1977). However, from the results of Lee *et al.* (1980), this initial efficiency was more likely associated with both bacterial and fungal growth on the litter. The

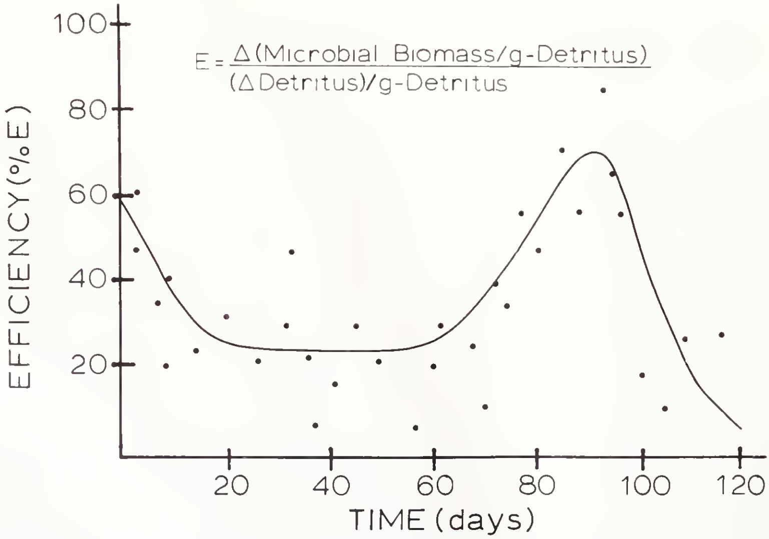

FIGURE 8. Instantaneous microbial conversion efficiencies of *Spartina alterniflora* litter biomass to microbially produced organic matter calculated from NH_4-N incorporation into litter. Plant biomass consumption was calculated from evolved CO_2-C (detritus is 48% C$\cdot$AFDW^{-1}).

peak efficiency of the secondary colonization is somewhat higher than the first and reflects the slightly higher optimal fungal growth efficiencies as compared to bacterial efficiencies. These results further indicate that fungi are the major decomposers in the latter stages of decomposition. However, Fallon and Pfaender (1976) reported 82 and 54% conversion efficiency values for fungal and bacterial communities, respectively, grown on *Spartina alterniflora* leachate. Their peak fungal conversion efficiency is somewhat higher than other reported values; nevertheless, their values are very close to the peak efficiencies measured in my study and suggest that initial decomposition is mediated by bacteria. The low conversion efficiencies of my experiment (the flat sections of Fig. 6), which are between 20 and 30%, are similar to conversion efficiencies for mixed fungal and bacterial populations (Fallon and Pfaender, 1976).

Microbial succession and biomass contribution during *Spartina alterniflora* litter decomposition is still an area of much controversy (for review, see Lee, 1980). These percolator observations indicate that fungi contribute a larger biomass to detritus than bacteria and this agrees with actual measurements of fungal biomass by S. Y. Newell (personal communication). One reason for this may be that the bacterial cell walls are much less recalcitrant than fungal hyphal components so that bacteria disappear after death while fungi remain.

The conclusion of this study is that the decomposition process can be successfully studied in well-controlled laboratory flow-through percolators. Not only was there good control over variables in these percolators but there was also low variability and complete recovery of chemical components. As a result, there was better resolution of changes in litter over time and it became apparent that nitrogen, and presumably microbially produced organic matter, increased in two steps. This could be caused by bacterial and fungal succession. One way to investigate the succession would be with antibiotics (Fallon and Pfaender, 1976). Biomass and successional information can also be obtained by concurrent direct measurements of bacteria (Daley and Hobbie, 1975) and fungi (Berg and Söderström, 1979) and with indirect biochemical measurements which relate to microbial biomass (Paul and Voroney, 1980; White *et al.*, 1980). Finally, the impact of meiofauna on this system can be checked by adding or removing these organisms from the litter and innoculum (Lee *et al.*, 1976).

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EFFECTS OF PRESSURE AND TEMPERATURE ON THE EKG AND HEART RATE OF THE HYDROTHERMAL VENT CRAB *BYTHOGRAEA THERMYDRON* (BRACHYURA)

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ABSTRACT

Effects of pressure and temperature on the electrocardiogram (EKG) and heart rate of crabs from the Galapagos deep-sea (2500 meters) hydrothermal vents were studied. Vent crabs require high hydrostatic pressure for long term survival. During decompression their EKG is disrupted and their heart rate is reduced. Low temperature reduces these decompression effects. The crabs have a higher temperature tolerance while at their environmental pressure (238 atm) and can withstand short-term exposure to temperatures as high as 37°C. Possible mechanisms for the action of pressure on neuromuscular systems are discussed. Habits and physiological capabilities of the crabs in the unusual vent environment are suggested on the basis of their physiological tolerances.

INTRODUCTION

More than 89% of the ocean bottom is at depths greater than 1000 meters. Yet, so little research has been done in the deep-sea that hydrothermal vents with their associated biological communities have only recently been discovered (Corliss *et al.*, 1979). The vent habitat, at a depth of 2500 meters, is characterized by high pressure, high biomass and variable, high temperatures. This contrasts with the bulk of the deep-sea which is also characterized by high pressue but has low biomass and stable low temperatures. By comparing aspects of the physiology of vent species and species adapted to the "typical" deep-sea it is possible to gain insight into the relative importance of certain environmental factors in selecting for physiological characteristics of deep-sea species. In addition, the influence of physiological characteristics on the distribution of deep-sea species can be studied.

This study investigates the adaptations of the hydrothermal vent crab, *Bythograea thermydron*, to the high pressure and fluctuating temperatures (Corliss *et al.*, 1979; Speiss *et al.*, 1980) of the vent environment to determine how these adaptations might affect the crabs' distribution in the deep-sea. This species is predominantly found in the mussel and pogonophoran beds in the warm vent water (up to 22°C, anoxic, up to 300 μM H_2S/l) but, they are found also at the periphery of vent areas where the water temperature is 2°C (110 μM O_2/l, no H_2S). Although few data are available on the temperature tolerance of other deep-sea species, the general belief is that they tolerate a range of only a few degrees (George, 1979a; Childress *et al.*, 1978).

Many studies have examined high pressure effects on shallow-living species (Rice, 1961; Bayne, 1963; Knight-Jones and Morgan, 1966; Macdonald, 1972;

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Wilcock *et al.*, 1978) but few have examined pressure effects in deep-sea species. In studies on deep-sea species, decompression caused reduced activity and loss of coordination, which are reversed by recompression (George, 1979a, b; Macdonald and Gilchrist, 1978; Menzies *et al.*, 1974; Yayanos, 1978). These studies suggest that disruption of nerve functions occur in some deep-living species when they are decompressed. The only study of pressure effects on the neuromuscular system of a deep-sea crustacean indicates that pressure effects occur below as well as above the species environmental pressure (Campenot, 1975).

We chose to study the electrocardiogram (EKG) and heart rate of *Bythograea thermydron* as indicators of pressure effects on the neuromuscular system. This approach was chosen for two reasons. First, if pressure affects either the heart innervation or the heart muscle itself this would be indicated by changes in the EKG or heart rate. Secondly, the technique could be done inside a steel pressure vessel and under the constraints imposed by operating aboard ship. All of the experiments in this report were carried out at sea, aboard R.V. Gilliss and R.V. New Horizon, during two expeditions to the Galapagos vents study sites (January–February and November–December 1979).

MATERIALS AND METHODS

Collection of specimens

Specimens of *Bythograea thermydron* were collected by the submersible *Alvin* from two vent areas: Mussel Bed (00°48.3′N, 86°09.1′W) and Rose Garden (00°48.9′N, 86°13.3′W). Baited traps were deployed and recovered by *Alvin* to capture the crabs. The traps were brought to the surface in an insulated container which protected the crabs from temperature changes but not pressure changes. The container was a 26 cm length of polyethylene pipe with an inside diameter of 30 cm and an outside diameter of 36 cm. The top and bottom were 5 cm thick polyethylene. A magnetic latch held the hinged top closed during ascent.

During the first expedition to the Galapagos hydrothermal vents, the captured crabs were kept at various temperatures and pressures because the environmental conditions permitting survival of this species were unknown. We changed our animal maintenance techniques as we gained information about the crabs requirements. The information collected during the first expedition showed us how to capture and maintain live crabs; during the second expedition we applied this knowledge in studies on the effects of pressure and temperature on their EKG and heart rate.

METHODS AND RESULTS

First expedition

The crabs captured during the first expedition were either maintained at 1 atm and temperature of 2°, 7°, 10°, or 12°C or at 120 atm and 2°C. The crabs at 1 atm were kept in aerated 1 gallon (3.79 L) containers inside small refrigerators. Individuals at 120 atm were kept in a transparent pressure vessel through which aerated, chilled (2°C ± 0.5°C) seawater was circulated by a high pressure pump (Quetin and Childress, 1980). This apparatus allowed us to make behavioral observations at different pressures but was limited to a maximum pressure of 120 atm which was lower than the crabs' environmental pressure. These studies showed that individual *B. thermydron* survived longer at 120 atm than at 1 atm. The mortality rate at 1 atm was high; among 64 crabs the maximum survival time was

5 days and it appeared to decrease with increasing temperature. In comparison, none of the 25 crabs kept at 120 atm and 2°C died during the remaining 21 days of the expedition. Behavior of the crabs differed at the two pressures. Their movements seemed spastic and uncoordinated at 1 atm and apparent loss of balance was common with many individuals falling on their backs with their legs splayed outward. In contrast, crabs at 120 atm were active and their behavior appeared similar to the behavior of crabs observed in the vent environment from the submersible. The low pressure effects on behavior were reversible by recompression. Furthermore, repeated decompression for short time periods, had no apparent long-term effect. One of the crabs survived for 18 months in our laboratory in a pressure vessel at 238 atm and 5°C, even though it was decompressed for 15 minutes every 10 days for feeding.

In summary, observations made during the first expedition indicate that *Bythograea thermydron* requires high pressure for survival. It was also found that temperature influences the effects of low pressure and that neuromuscular function in this species is disrupted at low pressures.

Second expedition

Crabs collected during the second expedition were maintained at their environmental pressure (238 atm) and 5°C except when they were decompressed for use in experiments. We used a high pressure aquarium system similar to the one mentioned above, except constructed of stainless steel, to keep the crabs at this pressure. The pressure vessel had a volume of 16 liters and was surrounded by a temperature controlled water jacket.

The temperature tolerance of individual *B. thermydron* was determined at their environmental pressure. These experiments were conducted in a small (6 l) stainless steel pressure aquarium system. A unique protocol was developed for determining the crabs temperature tolerance because they were unable to withstand high temperatures while at low pressure. Individuals were decompressed from 238 atm to 1 atm (2 atm/sec) and transferred from the maintenance vessel to the experimental vessel while both vessels were at 5°C. The pressure was increased to 238 atm after sealing the experimental vessel. The temperature was kept at 5°C for one hour and then was increased to the test temperature (at 0.2° to 0.3°C/min), slowing as the test temperature was reached due to equipment limitations. After one hour of exposure to the test temperature, the temperature was again reduced to 5°C (0.1°C/min), the crabs were decompressed and the mortality determined. Five crabs were tested at each of the following temperatures: 30°, 35°, 37°, or 40°C. All of the crabs in the groups exposed to 30° and 35°C (5 at each temperature) survived, but none in the 37.5° or 40°C groups survived the one hour exposure. These results showed that the vent crab could withstand a one hour exposure to temperatures as high as 35°C while at their environmental pressure of 238 atm.

The effects of pressure and temperature on the heart rate and EKG of individual *B. thermydron* was determined by implanting electrodes in the pericardial cavity. These electrodes were 22 gauge teflon-coated silver wire with the teflon insulation removed from the last 0.5 mm. The technique for implanting electrodes is as follows. An animal was removed from the maintenance vessel, secured with rubber bands to a piece of plexiglass and placed in a pan of cold seawater (approximately 2°C). A small hole for the recording electrode was drilled through the carapace over the heart and a hole for an indifferent electrode was drilled posterior to this, near the edge of the carapace. Electrodes were cemented into each of the holes with dental cement. This entire procedure took fewer than 15 minutes. The experimental crab, while still restrained, was placed in a pressure vessel at 5°C and the electrodes connected to electrical feedthroughs. The EKG signal was amplified and recorded on a high speed chart recorder.

Initial studies were performed to determine if the EKG and heart rate were stable after electrode implantation and if they were affected by the rate of compression and decompression. The short term effects of electrode implantation were investigated by recording the EKG for several hours immediately after recompression to 238 atm. The effects of compression and decompression were determined by recording the EKG during rapid changes in pressure. From these experiments we were able to determine the time course of the pressure effects.

The recordings showed no obvious short-term effects of electrode implantation. At 238 atm, the heartbeat was steady but the amplitude decreased with time (Fig. 1). Removal of the electrodes at the end of the experiments showed that material was deposited on them. This may have caused the amplitude decrease during the experiments by reducing the contact area of the silver electrodes with the body fluids. Neither a decompression rate of 24 atm/sec, nor a compression rate of 5 atm/sec affected the heart rate or the shape of the EKG form. When pressure effects were observed, they developed in fewer than 15 seconds after a change in pressure (Fig. 2).

To determine the effects of pressure and temperature on the EKG and heart rate of *Bythograea thermydron* the experimental animal was kept at 238 atm and 5°C for 30 minutes after electrode implantation and then subjected to a series of pressures in one of the two following orders: 238, 272, 340, 272, 204, 136, 68, 1, 68, 136, 204 and 238 atm or 238, 204, 136, 68, 1, 68, 136, 204, 272, 340, 272, and 238 atm. The maximum pressure of 340 atm was used because of the limitations

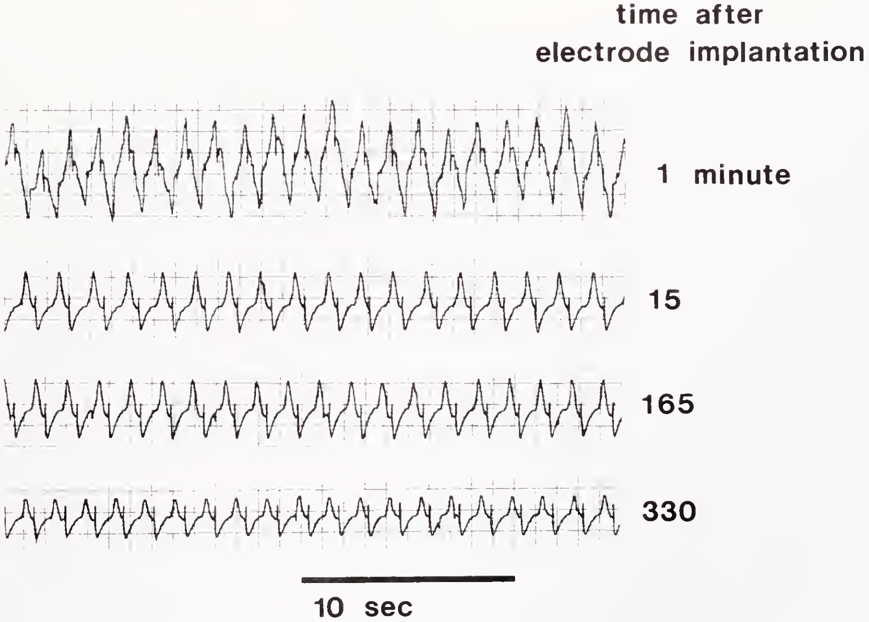

FIGURE 1. Time course for the stabilization of the EKG after electrode implantation in *Bythograea thermydron.*

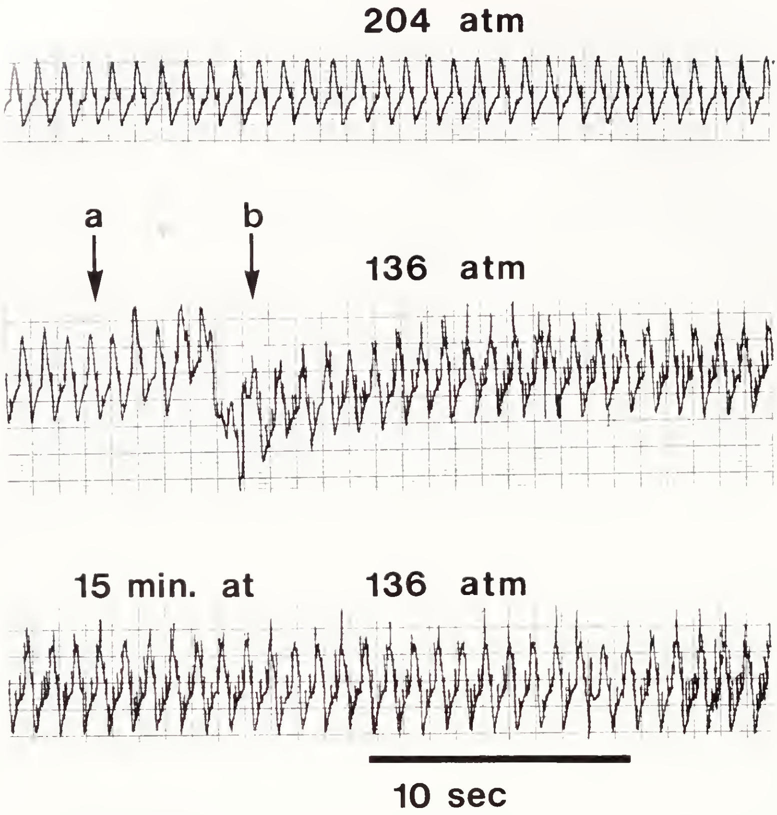

FIGURE 2. The time course for the development of the pressure effect in *Bythograea thermydron* at 12°C. a—immediately after decompression from 204 to 136 atm, b—development of the pressure effect (spikes overlying the normal EKG pattern).

of our equipment. Each crab was subjected to each pressure for 15 minutes during which the EKG was recorded for a 1 minute period every 3 minutes. Pressure changes were made at a rate of approximately 3 atm/sec. The crabs were tested sequentially at 3 temperatures, either in the order 5°, 12°, 20° or 5°, 20°, 12°C. Five individuals were examined.

Analysis of the EKG recordings emphasized changes in heart rate and EKG form. Changes in EKG amplitude were not considered in the analysis for two reasons. First, EKG amplitude was affected by the length of time that the recording electrodes were implanted in the experimental animal. Second, pressure caused small changes in the amplitude due to effects on the electrical connectors that penetrated the pressure vessel.

The results showed that the heart rate of the vent crab was not significantly ($P > 0.1$, t-test) affected by pressure over the range of 1 atm to 340 atm at 5°C (Fig. 3). At 12°C and 20°C the heart rate was significantly lower at 1 atm than at higher pressures ($P < 0.05$, t-test). Depression of the rate occurred at higher pressures at higher temperatures. At 12°C the heart rate slowed at pressures below 68 atm, while at 20°C it slowed below 136 atm. The form of the EKG trace also changed with pressure and temperature and showed interaction of these parameters.

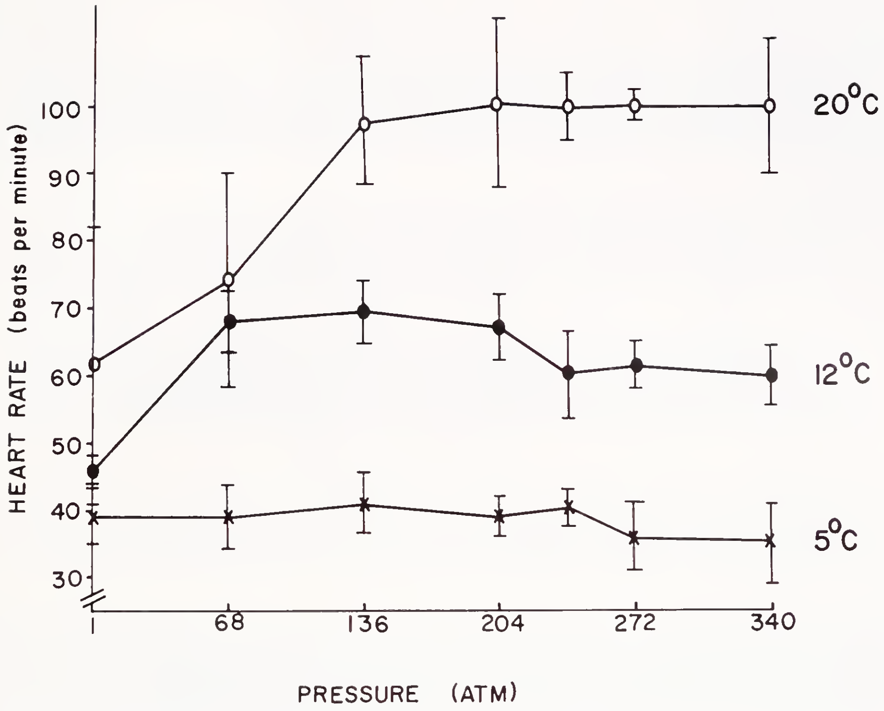

FIGURE 3. The effect of pressure and temperature on the heart rate of the crab, *Bythograea thermydron*. Error bars are ±1 S.E.

In this case, the pressure effect was characterized by random ($0.5 < P < 0.75$, goodness of fit test) spikes overlying the "normal" EKG (the EKG form at 238 atm) (Fig. 4). This pressure effect could not be attributed to an artifact of pressure and temperature effects on the recording electrodes because no electrical activity was recorded from electrodes placed in the pressure vessel alone or implanted in dead crabs exposed to the same experimental conditions. The disruptive effects of reduced pressure on the EKG form occurred at higher pressures at higher temperatures. At 5°C the disruption occurred at pressures between 1 atm and 68 atm, at 12°C it occurred between 68 atm and 136 atm and at 20°C it occurred at 136 atm to 204 atm. At the crabs environmental pressure of 238 atm, a temperature of 30°C disrupted the heartbeat (Fig. 5) and three of the five experimental animals died within two hours.

The time course for the development of pressure effects was not significantly different at the three temperatures tested (mean ± 1 S.E.: 5°C, 7.9 ± 1.61; 12°C, 9.00 ± 2.58; 20°C, 9.67 ± 1.20). These values were determined after a 68 atm drop in pressure and are the times from the end of the pressure change till the first appearance of at least 4 random spikes in each heartbeat. These are estimates for the time of development of pressure effects because it is unknown exactly when the effect began during the 15 second decompression. Further study will be required to determine the exact time course for decompression effects and also to determine the time course for the reversal of these effects during recompression. Reversal of decompression effects, however, appears to be dependent on time spent at low pressure.

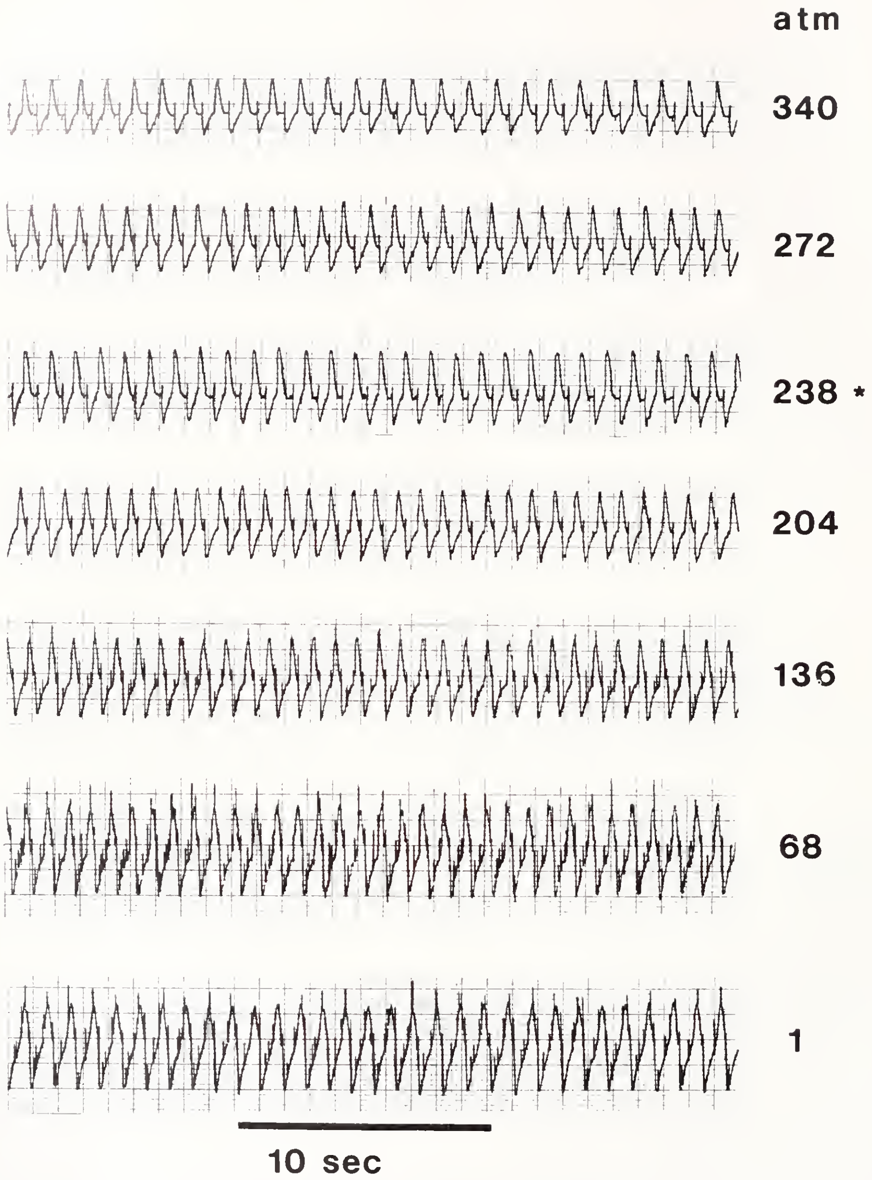

FIGURE 4. The effect of pressure on the EKG form of the crab *Bythograea thermydron* at 12°C; (*) the pressure normally experienced by the crab in its environment.

DISCUSSION

The disruption of the hydrothermal vent crabs electrocardiogram (EKG) during decompression is the first direct evidence of low pressure effects on the neuromuscular system of a deep-sea species. The increased electrical activity of the heart muscle is perhaps indicative of effects throughout the neuromuscular system which may account for the crabs abnormal behavior and eventual death at low pressures.

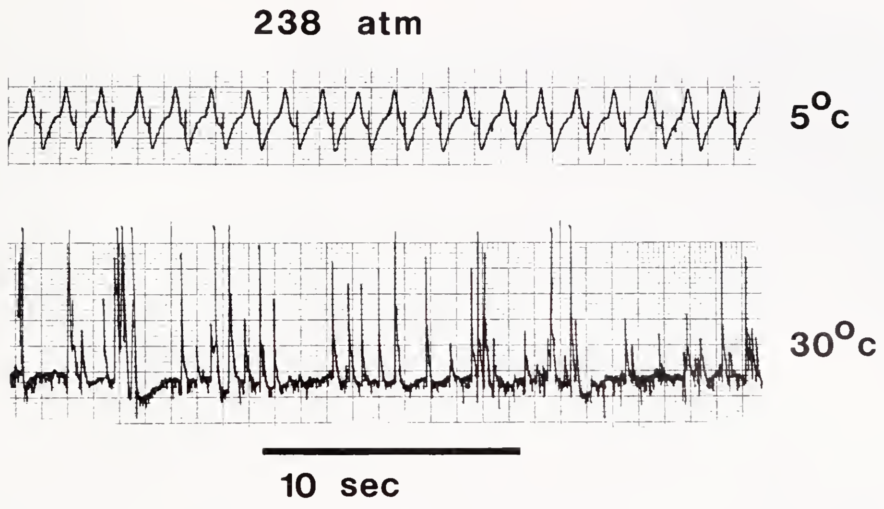

FIGURE 5. The disruptive effect of high temperature on the EKG form of *Bythograea thermydron.*

Deep-sea amphipods show similar behavior, indicative of neuromuscular effects, when they are decompressed. They display jerky pleopod movements and have difficulty in initiating pleopod rhythm when decompressed to half their environmental pressure (Yayanos, 1981).

Recent investigations provide evidence for several mechanisms by which pressure could affect the neuromuscular functioning of deep-living species. One mechanism may act through effects on synaptic transmission (Campenot, 1975). In the moderately deep-living (300–1600 meters) crab, *Geryon quinquidens*, high pressure depresses the excitatory junctional potential (ejp) amplitude in muscle fibers (Campenot, 1975). Excitatory junctional potentials reflect the local response of muscle membrane to transmitter released by nerve endings. Frequency of nerve stimulation is one of the controlling factors of ejp amplitude and increases in stimulation frequency lead to greater ejp amplitude through increases in transmitter release (Dudel and Kuffler, 1961; Frank, 1973). When ejp amplitudes are great enough, muscle fibers contract. Campenot (1975) attributes the high pressure depression of ejp amplitude in *Geryon* to interference with the release of transmitter substance. If the same mechanism operates in vent crabs then the reverse effect, produced by reduced pressure, might also occur. If this is the case, release of transmitter at low pressures would be increased and greater ejp amplitudes would occur for a given frequency of nerve stimulation. Under these conditions, some nerve impulses which produce subthreshold ejp amplitudes for muscle contraction at high pressure would produce larger ejp amplitudes and therefore muscle contraction at low pressures. This could be responsible for the increased electrical activity in the EKG recordings.

Another mechanism by which pressure may affect the neuromuscular system of vent crabs is through changes in cell membranes. Membrane structure changes could be responsible for permeability changes leading to changes in nerve excitability. Brauer *et al.*, (1980) have shown that deep-living freshwater amphipods go into negative ion balance at 1 atm and Johnson and Miller (1975) have shown that pressure affects the permeability of model membranes to some ions. In addition, pressure induced changes in nerve membrane conductance have been demonstrated in both shallow (Spyropoulos, 1957; Wann *et al.*, 1979) and deep-living species (Campenot, 1975). The mechanisms by which these effects operate are not yet understood but one possibility is that pressure can cause viscosity changes and phase transitions in membrane lipids (Yayanos *et al.*, 1978). Since high pressure is expected to have an ordering effect on membrane bilayer structure (Boggs *et al.*, 1976) it seems reasonable that decreased pressure would reduce the ordering and lead to increased membrane conductance. Additional evidence that membrane lipids may be involved comes from investigations on enzymes that show nonlinear Arrhenius plots. Ceuterick *et al.* (1978) have shown that lipids are responsible for breaks in the plot of *Azotobacter* nitrogenase and that the temperature at which the break occurs increases with increasing pressure. The results of another study indicate that Na^+/K^+-ATPase activity decreases with increasing pressure (De Smedt *et al.*, 1979). Plots of the ATPase activity versus pressure show a breakpoint that increases with increasing temperature. This agrees with pressure shifts for melting transitions in phospholipids and aliphatic chains. De Smedt *et al.* conclude that an aliphatic chain melting process is involved in the pressure dependence of Na^+/K^+-ATPase.

It is possible that none of the mechanisms mentioned above are responsible for the observed pressure effects. Effects on reaction rates and enzyme catalytic properties due to pressure effects on molecular volume changes could explain the observed changes both in synaptic transmission and in ion distribution. For example, synaptic transmission could be affected by a change in the rate of association of transmitter substance with the receptor on the post synaptic membrane (Akers and Carlson, 1976). Ion balance would be influenced if membrane bound ion transporting ATPases were affected (Pequeux and Gilles, 1977; Goldinger *et al.*, 1978; Hall, 1979). In the vent crab, however, pressure effects appear to involve a structural change rather than a kinetic or equilibrium change. This hypothesis is supported by two results. First, the time course for decompression effects on the EKG are rapid; frequently less than 10 seconds. Second, temperature does not alter the time course of the effect. If reaction rates or equilibria were involved then the time course would be influenced by temperature. Results of investigations on enzymes of deep-living fish further suggest that pressure effects on enzymes do not cause the low pressure effects observed in the vent crabs. The enzymes of deep-living fish are relatively insensitive to pressure in comparison to those of shallow species (Siebenaller and Somero, 1979).

Pressure affects the vent crabs heart rate as well as EKG and may reflect effects on the cardiac pacemaker. Pressure effects on pacemaker cells have been demonstrated in some mammalian heart preparations (Örnhagen and Hogen, 1977) and also in the rhythmically firing cells of *Helix* (Wann *et al.*, 1979). The exact effects of pressure on the heart rate of deep-living species is unclear. Decompression causes an increase in heart rate in the deep-sea ostracod *Gigantocypris mulleri* (Macdonald, 1972) but in the mysid *Gnathophausia zoea* heart rate increases with compression. The influence of temperature on pressure effects on the heart rate of vent crabs is similar to that on the EKG. Lower temperatures reduce the effects of decompression.

The influence of temperature on pressure effects has been noted in other studies but in some cases the results conflict (Napora, 1964; Teal and Carey, 1967; Johnson and Eyring, 1970; Gillen, 1971; Childress, 1977; George, 1979b). This may be caused by several factors. First, pressure affects many levels of organization of biological systems and may affect some more than others depending on the species. Second, pressure effects may be increased, decreased or nullified by temperature depending on the mechanisms by which pressure acts. Conflicting data on pressure effects also comes from early studies on shallow freshwater and marine organisms. Organisms were subjected to pressures far outside their normal range in these studies and little regard was given to temperature (Fontaine, 1930; Ebbecke, 1935; review by Gordon, 1970). In more recent investigations, pressures and temperatures within the environmental ranges of the species tested were used. These studies show that pressure generally has little effect (Teal and Carey, 1967; Pearcy and Small, 1968). When pressure effects occur, they are slight and are counteracted by low temperature (Napora, 1964; Teal, 1966, 1971). The experimental results on vent crabs agree with these latter studies. Although pressures below 68 atm apparently lead to disruption of the vent crabs neuromuscular functioning which eventually is lethal, no effects occur over the range of 136 to 340 atm. In general, vent crabs are only affected by pressures far outside their habitat range.

The temperature and pressure tolerance characteristics of vent crabs provide insight into their habits in the vent environment. The crabs high temperature tolerance and ability to live over a wide temperature range is extremely different from the limited temperature tolerance of other deep-sea species and species living at stable low temperatures in the antarctic (McWhinnie, 1964; Somero and DeVries, 1967; George, 1979a, 1979b). The crabs utilize this capability and are found throughout the vent environment. They have been observed in the warmest water exiting the vents (22°C) and also at the periphery of the vent environment where the temperature is 2°C. Their ability to withstand low temperature implies that they may be able to escape a dying vent or one showing increased thermal activity by leaving the vent habitat. The observed distribution of greater numbers of crabs in the warm water, therefore, probably reflects ecological factors and not physiological limits. The greatest biomass and consequently the greatest amount of potential food is near the warm water. Vent crabs have been observed eating pieces of vestimentiferan worms, which are most abundant in the warm water. Unlike vent crabs, distributions of other species around the vents may be influenced by the high temperatures. Most vent species appear to be endemic and relatively few non-vent deep-sea species are found near the Galapagos vents. Non-vent species may be restricted by their temperature tolerances from taking advantage of the vent environments high biomass.

The distribution of vent crabs apparently is not limited by temperature but it may be influenced by pressure. They may be limited to depths greater than 680 meters due to disruption of their neuromuscular functioning at pressures below 68 atm. The crabs upper lethal pressure limit was not determined due to limitations of our equipment but no high pressure effects were observed at pressures up to 340 atm, suggesting that the crabs could live at depths of at least 3400 meters. However, it is important to note that a species pressure tolerance may not be a reliable indicator of their depth distribution. This is mainly because the relative importance of genetic factors versus acclimation on pressure tolerance is not yet understood. Studies show that individuals of the same species, collected from different depths, may have different pressure tolerances (George, 1979a). Also, the pressure tolerance of even shallow-living species can be slightly increased by acclimation to elevated pressures (Avent, 1975). The temperature and pressure tolerance characteristics of vent crabs suggest that they are not limited by these environmental parameters to the vent habitat. They also are not limited by a high food requirement because their metabolic rate is comparable to that of other deep-sea crustaceans (Mickel and Childress, in prep.). Since the crabs apparently are not restricted to the vent environment by their physiological characteristics, their distribution is presumably the result of behavioral patterns evolved in response to selection by ecological factors.

These studies on the vent crab may provide insight into factors which result in the failure of many groups of animals to penetrate into the deep-sea. For example, Wolff (1961) states that of a total of approximately 3500 brachyuran crab species only about 125 are found at depths greater than 200 meters. Brachyuran crabs apparently are not limited by physical characteristics of the deep-sea because the vent crabs have evolved physiologically to live at high pressures and low temperatures. Since the metabolic rate of vent crabs at low temperatures appears to be comparable to other deep-sea crustaceans, (caridian shrimps) which as a group live to much greater depths, metabolic rate adaptation also does not appear to be a limit. It seems likely, therefore, that the failure of brachyuran crabs to extensively inhabit the deep-sea is not due to an inability to evolve the necessary physiological abilities, but rather may be attributed to ecological factors which select against the brachyuran body form in most of the deep-sea. This suggests that, in general, the depth limits of taxa in the deep-sea may not be set by an inability to evolve appropriate physiological characteristics, but rather by the failure of a particular body form to be effective in the ecological milieu of the deep-sea.

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HOST ENHANCEMENT OF SYMBIONT PHOTOSYNTHESIS IN THE HYDRA-ALGAE SYMBIOSIS

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ABSTRACT

Photosynthesis by the *Chlorella*-like algal symbiont of the green hydra, *Hydra viridis*, was determined for algae *in situ* and *in vitro* by measuring photosynthetic oxygen evolution in a modified polarographic electrode. Effects of light intensity, environmental oxygen concentration, and bicarbonate ion concentration on photosynthetic oxygen evolution were measured, with the following results: (1) Bicarbonate ion increased photosynthesis by algal symbionts *in situ* with up to 1 m*M* bicarbonate added. (2) Based on light intensity/photosynthesis data, photosynthetic oxygen evolution in symbionts *in situ* was greater than in those *in vitro*, especially at ambient oxygen concentrations. Oxygen severely inhibited photosynthetic oxygen evolution by symbionts *in vitro* but had little or no effect on algae *in situ* up to ambient oxygen concentrations. These data suggest that hydra symbionts gain a significant photosynthetic advantage, especially at ambient ($=8$ mg l^{-1}) oxygen concentrations, when they are associated with their hosts. The role of the host-symbiont relationship in contributing to this advantage is discussed.

INTRODUCTION

The green hydra, *Hydra viridis*, harbors *Chlorella*-like symbionts within its gastrodermal cells. These photosynthetically active algae provide the host with photosynthetically fixed carbon, mainly in the form of maltose (Muscatine and Lenhoff, 1963; Muscatine, 1965), which augments the hydra's nutrition under starvation conditions (Muscatine and Lenhoff, 1965; Pardy and White, 1977). The nutritional advantage of the symbiosis to the host is clear. The advantages of the association to the algae are not so evident.

Oxygen inhibition of photosynthesis in *Chlorella* was first described by Warburg (1920). Oxygen may inhibit photosynthesis by oxidizing photosynthetic components, by decreasing reducing equivalents needed for carbon fixation, and by competing with CO_2 at the carboxylating enzyme, ribulose 1,5 bisphosphate carboxylase/oxygenase. This last mechanism leads to the phenomenon known as photorespiration, the light stimulated uptake of O_2 and release of CO_2 (Goldsworthy, 1970; Chollet and Ogren, 1975; Tolbert, 1979; Zelitch, 1979). Photorespiration does not conserve net energy and results in a net loss of carbon.

Downton *et al.* (1976) determined that increasing oxygen concentration substantially inhibited oxygen evolution by *Tridacna* symbionts *in vitro*, but presented no data on the effects of oxygen on symbionts *in situ*. If oxygen also inhibits photosynthesis in hydra symbionts, algae within the host might realize a photo-

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Abbreviations: 3-(3-4-dichlorophenyl)-1,1-dimethylurea, DCMU; rate of photosynthesis at light saturation, P_{max}; light intensity at onset of light saturation, I_k; respiratory quotient, R.Q.; substrate concentration at which rate of enzymatic reaction is half maximal, K_m; photosynthetically active radiation, PAR.

synthetic advantage. Decreased oxygen and increased CO_2, conditions expected within respiring host tissues, would tend to favor photosynthesis. We examined hydra algae *in situ* and *in vitro* to determine the magnitude of O_2 inhibition of photosynthesis, and the effects of light and exogenous CO_2 on photosynthetic oxygen evolution. *In vitro* experiments required isolation of symbionts from their host and examination of their photosynthetic performance in an artificial medium. Such an approach has proved fruitful in previous studies of symbiotic associations such as in the release of maltose by hydra symbionts (Muscatine, 1965), the release of photosynthate from zooxanthellae (Trench, 1971a) and the effects of host homogenate on the release of glycerol by zooxanthellae (Trench, 1971b). Such analysis always risks the introduction of effects specific to the artificial environment devised for the symbionts, but as yet no worker has been able to deduce or duplicate exactly the conditions occurring within any host organism, so this risk is true of any system used.

MATERIALS AND METHODS

Experimental organisms

Green hydra (*Hydra viridis*, Florida strain) were mass-cultured in M solution and fed daily with brine shrimp (*Artemia salina*) nauplii, as described by Lenhoff and Brown (1970), except that the M solution contained no bicarbonate. Cultures were maintained at 20°C in a photoperiod incubator (Freas 818, Precision Scientific Co.), set for constant illumination by a single 40-watt cool-white fluorescent tube (Sylvania F48T10). Quantum flux at the center of the incubator was 60 $\mu E \cdot m^{-2} \cdot s^{-1}$ of photosynthetically active radiation (PAR) as measured with a Licor LI-170 quantum sensor (Lambda Instruments). Animals were not fed for 24 h before they were used in experiments.

Oxygen measurements

A slide projector with a 500-watt lamp (Sylvania DAY-DAK Tungsten) and infrared filter provided light for photosynthesis experiments (Fig. 1). A series of mirrors directed the projector beam to a collimating lens. This lens focused light onto the surface of a Plexiglas light guide inserted into the chamber of a Rank Brothers oxygen electrode (Rank Brothers, Cambridge, England). Light intensity was regulated using neutral density filters (Bausch and Lomb) for coarse adjustment and a diaphragm for fine adjustment. Light intensity at the base of the light guide was measured using a Licor quantum sensor. The spectral emission of the projector lamp was analyzed using an Isco scanning spectroradiometer (Model SR, Instrumentation Specialties Co.) (Fig. 2). The O_2 concentration in the chamber was recorded continuously by connecting the electrode to a chart recorder (Fisher Recordall 5000, Fisher Scientific Co.). The chamber was maintained at 20°C with a circulating, temperature-controlled water bath (Markline 2095, Forma Scientific Co.).

Sodium dithionite ($\simeq$5 mg) added to 5 ml of distilled water in the electrode chamber calibrated the electrode at zero O_2 concentration. An IBC dissolved oxygen meter (Model 500-051, International Biophysics Inc.) was used to determine the O_2 concentration in air-saturated M solution ($\simeq$8.4 mg O_2 l^{-1}) which was used to set the span of the electrode.

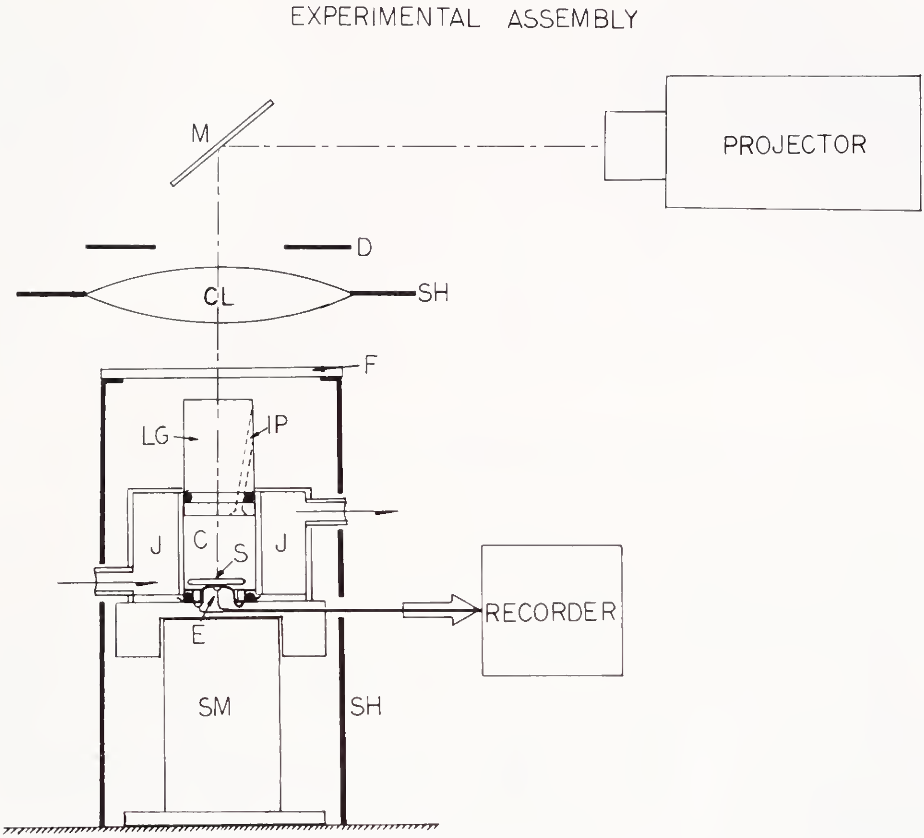

FIGURE 1. Apparatus used to measure photosynthetic oxygen evolution. M = mirror assembly; CL = collimating lens; D = diaphragm; SH = stray light shield; F = neutral density filter(s); LG = light guide; IP = injection port; C = sample chamber; S = stir bar; J = water jacket; E = polarographic oxygen electrode; SM = stirring motor.

Photosynthesis of H. viridis *symbionts* in situ

About 150 hydra were used to determine O_2 evolution by algal symbionts *in situ*. Carryover of contaminants with the animals was minimized by washing them with fresh M solution before they were placed in the oxygen electrode chamber. Animals were placed in a small basket and suspended in 5 ml of fresh M solution (pH 7.5). $KHCO_3$ was added to a final concentration of 1.0 m*M* in all experiments with intact hydra except those designed to test the effect of this compound on symbiont photosynthesis (our M solution contained about 0.140 m*M* HCO_3^- from atmospheric CO_2). The addition of $KHCO_3$ assured that the symbionts were not CO_2 limited during illumination at higher light intensities. Oxygen evolution was determined over a 5–10 min period and the rate of oxygen evolution determined from these data. Light intensity-photosynthesis curves were constructed by varying the light intensity impinging on the chamber as previously described with 1.0 m*M* $KHCO_3$ present in the chamber. The effects of $KHCO_3$ on O_2 evolution were determined by varying this parameter at a fixed light intensity, and the effects of

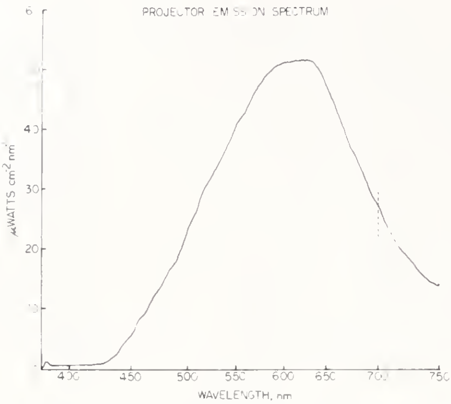

FIGURE 2. Emission spectrum of projector lamp as determined with a scanning spectroradiometer. Dotted lines define range of photosynthetically active radiation (P.A.R.).

oxygen on O_2 evolution were determined by varying O_2 concentration at 1 m*M* $KHCO_3$ and a fixed light intensity.

Photosynthesis of H. viridis *symbionts* in vitro

Symbionts for *in vitro* studies were isolated from hosts by homogenizing approximately 150 hydra using a Potter Elvenjeim tissue homogenizer with a Teflon pestle in 100 m*M* potassium phosphate buffer (pH 6.4) containing 1 m*M* $MgSO_4$ and 1 m*M* $CaCl_2$. Algae were pelleted from the homogenate using a tabletop centrifuge at 1700 × *g* for 1 min. The algal pellet was washed 6 times and finally resuspended in 5 ml of homogenization buffer. Buffer was added to yield a concentration of approximately 2×10^7 algal symbionts in a 5 ml sample. This concentration yielded maximum photosynthesis by the isolated symbionts as determined in this laboratory (data not shown). A 5 ml aliquot was placed in the electrode chamber, and oxygen concentration was adjusted by bubbling with nitrogen. $KHCO_3$ added to the chamber to a final concentration of 12 m*M* yielded sufficient CO_2 to prevent CO_2 limitation at higher light intensities. The electrode chamber was sealed with the Plexiglas light guide and measurements of oxygen evolved were made for 5–10 min in the light. Rates of oxygen evolution were determined from these data.

Chlorophyll content

Following O_2 measurements on intact hydras, animals were homogenized as described previously. Total chlorophyll (Chl) content of symbionts both *in situ* and *in vitro* was assayed using a 10 μl sample of suspension extracted in 3 ml of absolute methanol. Fluorescence of the methanolic extracts was measured in a Turner III Fluorimeter (Turner and Associates) using a 5-60 primary filter and a 2-64 secondary filter (Turner and Associates). The fluorimeter was calibrated using chlorophyll standards prepared from methanolic extracts of symbionts and analyzed for total chlorophyll as described by Mackinney (1941), using a Spectronic-20 spectrophotometer. The 150 hydra used in experiments yielded about $1.5–3.0 \times 10^{-2}$ mg chlorophyll and algal suspensions were adjusted to yield 1.0×10^{-2} mg Chl.

Rates of oxygen evolution

Rates of O_2 evolution for symbionts *in situ* were determined by subtracting oxygen consumption in the dark from O_2 evolution in the light. For algal suspensions, dark respiration was not subtracted from rates observed in the light. In light, dark respiration is suppressed in algae (Brown and Tregunna, 1967). Furthermore, respiration in *Chlorella* has been shown to be less than 10% of the rate of photosynthesis (Brown and Tregunna, 1967), and we have found that symbionts *in vitro* also exhibit rather low rates of dark respiration. These rates were so close to zero at 1–2 mg O_2 l^{-1} that they were not accurately measurable with our apparatus for the most part. The *Hydra viridis* respiration rate averaged about 0.53 μmoles $min^{-1} \cdot mg\ chl^{-1}$, much greater than that observed for the algae alone. We therefore felt justified in ignoring the respiratory component of the algae in measuring oxygen evolution by whole animals.

RESULTS

The effects of exogenous bicarbonate on photosynthesis by hydra symbionts in situ

Adding HCO_3^- ions to the medium strongly affected photosynthetic oxygen evolution by *in situ* symbionts of *H. viridis* (Fig. 3). Oxygen evolution by the symbionts increased sharply with up to 1 m*M* added bicarbonate, followed by a much slower increase on further additions.

The effects of incident light intensity on photosynthesis by hydra symbionts

Photosynthetic O_2 evolution by *H. viridis* symbionts *in situ* and *in vitro* increased with increasing incident light intensity (Fig. 4, 5). However, photosynthesis by the symbionts was dramatically influenced by the O_2 concentration of the medium and whether the symbionts were *in situ* or *in vitro.* At O_2 concentrations ranging from 0.5–1.5 mg O_2 l^{-1} (Fig. 4) rates of O_2 evolution *in vitro* were only slightly lower than those *in situ.* In contrast, at an O_2 concentration of 6.5–7.0 mg l^{-1} (Fig. 5) rates of oxygen evolution by symbionts *in vitro* were much lower than those of symbionts *in situ* at all light intensities examined.

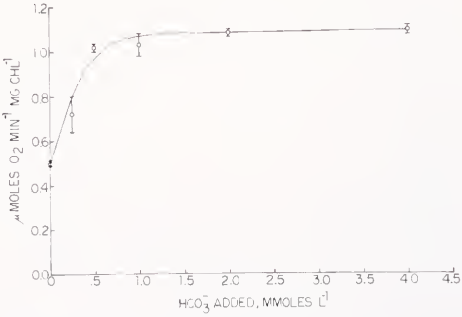

FIGURE 3. The effect of added bicarbonate on oxygen evolution by *H. viridis* algal symbionts *in situ.* $P_{max} = 11.9 \times 10^{-4}$ mmoles O_2 min^{-1} mg Chl^{-1}. Hydra were incubated in M solution, pH 7.5, which was purged with N_2 to an oxygen concentration of 1.5–2.0 mg O_2 l^{-1}. Circles = mean values. Vertical bars = S.D. n = 2 to 4. Each point = integrated rate of O_2 evolution over 5–10 min period.

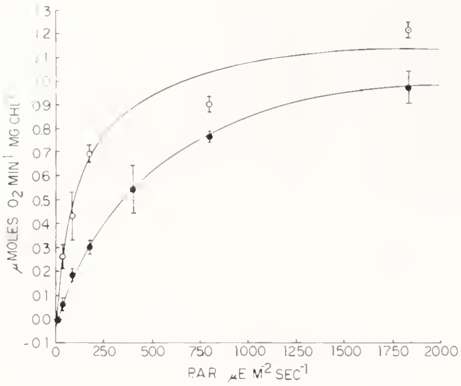

FIGURE 4. Oxygen evolution as a function of light intensity by symbionts of *H. viridis* at low (0.5–1.5 mg l^{-1}) oxygen concentrations. Open circles = algal symbionts *in situ*, hydra maintained in M solution + 1 m*M* $KHCO_3$. Closed circles = algal symbionts *in vitro* in phosphate buffer, pH 6.4 + 12 m*M* $KHCO_3$. Circles = mean values. Vertical bars = S.D. n = 2 to 4. Each point = integrated rate of O_2 evolution over 5–10 min period.

The light intensity-photosynthesis curves were analyzed on the basis of: the rate of photosynthesis at light saturation, P_{max} (maximal rate of photosynthesis); the light intensity at the onset of light saturation, I_k (Talling, 1957; Yentsch and Lee, 1966); and the slope of the light-limited portion of the curves (Yentsch and Lee, 1966). At O_2 concentrations near ambient (6.5–7.0 mg l^{-1}) the P_{max} of symbionts *in vitro* was almost 95% lower than that of those *in situ* (Table 1). In addition, the slope of the curves in the light-limited region decreased substantially for symbionts *in vitro* compared to those *in situ*, increasing I_k about twofold in isolated algae. In contrast, at lower O_2 concentrations (0.5–1.5 mg l^{-1}) symbionts *in vitro* had a slightly lower P_{max} (15%) than those *in situ*, while the slope of the curve during light limitation decreased about the same as at near ambient O_2 concentrations. This increased I_k about fourfold.

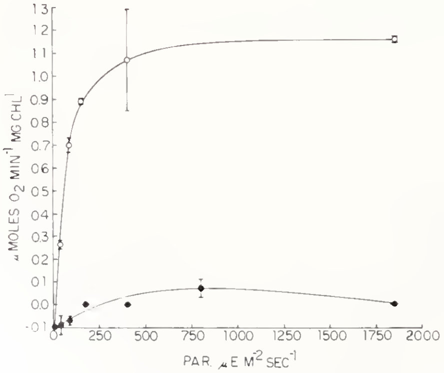

FIGURE 5. Oxygen evolution as a function of light intensity by symbionts of *H. viridis* at near ambient (6.5–7.0 mg l^{-1}) oxygen concentrations. Open circles = algal symbionts *in situ*, hydra maintained in M solution + 1 m*M* $KHCO_3$. Closed circles = algal symbionts *in vitro* in phosphate buffer, pH 6.4 + 12 m*M* $KHCO_3$. Circles = mean values. Vertical bars = S.D. n = 2 to 4. Each point = integrated rate of O_2 evolution over 5–10 min period.

TABLE I

Kinetics of oxygen evolution as a function of light intensity for algae of H. viridis. *Slope is that of initial region of curves in Figures 4 and 5.*

O_2 Conc.	Symbiont location	P_{max} mM O_2 min^{-1} mg chl^{-1}	I_k μE m^{-2} s^{1}	Slope
6.5–7.0 mg O_2 l^{-1}	*in situ*	1.16	125	13
	in vitro	0.06	280	0.67
0.5–1.0 mg O_2 l^{-1}	*in situ*	1.13	125	13
	in vitro	0.96	520	2.4

Thus, photosynthetic response to light decreased in algal symbionts isolated from their hosts, and this decrease was greater at higher O_2 concentrations. In contrast, the photosynthetic response to light of symbionts *in situ* was relatively insensitive to oxygen.

Oxygen inhibition of photosynthetic oxygen evolution

As the light intensity-photosynthesis data suggested, whether the symbionts were *in situ* or *in vitro* strongly influenced the inhibition of photosynthetic oxygen evolution by O_2 (Fig. 6). Increasing environmental O_2 concentration from low to near air-saturation strongly inhibited O_2 evolution by symbionts *in vitro.* In contrast, symbionts *in situ* were only slightly affected, and only at the higher O_2 concentrations. This is evident from percent inhibition of O_2 evolution as a function of O_2 concentration (Fig. 7). For example, at 8 mg O_2 l^{-1} symbionts *in vitro* were inhibited 110%, but symbionts *in situ* only 11%.

DISCUSSION

The Rank Brothers polargraphic oxygen electrode has been used to study oxygen evolution by a coral and its symbiotic zooxanthellae (Crossland and Barnes, 1977) and by cell suspensions of photosynthetic organisms (Findenegg, 1976; Burris, 1977; Takabe and Akazawa, 1977). Our use of the Plexiglas light guide avoids the len-

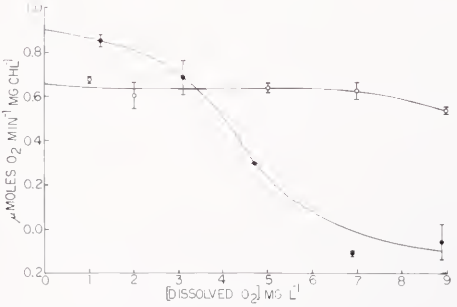

FIGURE 6. Oxygen evolution by *H. viridis* symbionts as a function of oxygen concentration. Open circles = algal symbionts *in situ*, hydra incubated at 400 μE·m^{-2}·s^{-1} in M solution + 1 mM $KHCO_3$. Closed circles = algal symbionts *in vitro* in phosphate buffer, pH 6.4 + 12 mM $KHCO_3$ at 600 μE·m^{-2}·s^{-1}. Initial oxygen concentrations were produced by N_2 purging. Circles = mean values. Vertical bars = S.D. n = 2 to 4. Each point = integrated rate of O_2 evolution over 5–10 min period.

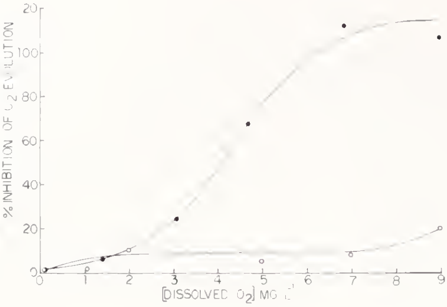

FIGURE 7. Percent inhibition of *H. viridis* symbiont oxygen evolution by oxygen. Open circles = algal symbionts *in situ*. Closed circles = algal symbionts *in vitro*. Each point represents means of Figures 5 and 6.

ticular effects of the electrode's cylindrical jacket and chamber walls, which otherwise increase or decrease the light actually reaching organisms in the chamber. The light guide evenly illuminates small animals such as hydra. Finally, with our modifications, light intensity measurements are easier, requiring only a 2π light sensor.

Symbionts were isolated in phosphate buffer to avoid organic buffering systems the symbiotic algae might metabolize. For instance, *Chlorella pyrenoidosa* will photometabolize acetate (Goulding and Merrett, 1966). Other workers have used phosphate-citrate buffer with algal symbionts (Cernichiari *et al.*, 1969) but have presented no data concerning its possible metabolism. The ion concentrations used in our buffer were derived empirically; the pH was adjusted to 6.4 because this was found to give the maximum oxygen evolution at a given light intensity. This pH also yields roughly equal concentrations of CO_2 and HCO_3^- in solution.

Symbionts *in situ* carried out a basal rate of photosynthetic O_2 evolution without added exogenous bicarbonate. Under these conditions the association consumed O_2 at a rate of 0.53 μmol $O_2 \cdot min^{-1} \cdot mg\ chl^{-1}$, which we attributed to host respiration. The R.Q., defined as the ratio of the CO_2 produced to O_2 consumed by an organism (Richardson, 1929; Kleiber, 1961), may be used to calculate the CO_2 production of an organism if its O_2 consumption is known. Pardy and White (1977) calculated the R.Q. for hydra unfed for 24 h at 0.862 at ambient O_2 concentrations. This value gives an estimated CO_2 output of 0.46 μmol $CO_2 \cdot min^{-1} \cdot mg\ chl^{-1}$. Assuming that CO_2 fixed into carbohydrates is the main sink for reducing power generated by photolysis of water, a mole of O_2 should be evolved from the symbionts for every mole of CO_2 taken up. Thus, under conditions where the sole source of CO_2 for symbiont photosynthesis is the host respiration, oxygen evolution by the symbionts *in situ* should be about 0.46 μmol $O_2 \cdot min^{-1} \cdot mg\ chl^{-1}$. The slight discrepancy between this value and the observed O_2 evolution of 0.50 μmol $O_2 \cdot min^{-1} \cdot mg\ chl^{-1}$ may be attributed to O_2 evolution by symbionts due to non-CO_2 fixing electron flow (Anderson, 1978) or to the small amount of CO_2 remaining in the M solution after purging with N_2.

Thus, with little or no CO_2 available externally, the rate of photosynthesis of hydra symbionts depends on the rate at which the host generates CO_2 internally, provided the association is not light-limited. Under such conditions the symbiotic association will not show net oxygen production, as an R.Q. greater than 1 would be required of host metabolism. Pardy and Dieckmann (1975) probably were unable to show net O_2 evolution in *H. viridis* for this reason. Reisser (1980) reached a similar conclusion.

However, when bicarbonate is available in the medium, algal symbionts are able to use the added CO_2 to photosynthesize. They are mainly limited by other factors, such as light intensity, and not by host CO_2 production. This is shown by the 120% increase we observed with the addition of as little as 1 m*M* $KHCO_3$. Reisser (1980) reported similar increases for the *Paramecium/Chlorella* symbiotic association upon additions of bicarbonate. Net O_2 evolution from the *Hydra/Chlorella* symbiotic association occurred under these conditions. We have observed light compensation for the intact association as low as 175 $\mu E \cdot m^{-2} \cdot s^{-1}$, a little less than one-tenth full sunlight. Above this value the association produces oxygen rather than consumes it. Thus, in ecological terms, when light and CO_2 are abundant, the green hydra symbiotic association becomes a producer. The hydra is, in effect, a carnivorous plant.

Summarizing, when the environmental concentration of CO_2 is low, photosynthesis by hydra symbionts depends on, and is therefore modulated by, host CO_2 evolution at moderate light intensities. In contrast, when environmental CO_2 is abundant, the rate of photosynthesis is limited by other factors, such as light.

Rates of photosynthetic O_2 evolution for hydra symbionts *in situ* were generally lower than those for other symbiotic or free-living *Chlorella.* Webb *et al.* (1980) reported a rate of oxygen evolution for *Chlorella vulgaris* five times the values that we observed for *H. viridis* symbionts measured *in situ.* Similarly, Reisser (1980) reported a rate of O_2 evolution for *Paramecium* symbionts *in situ* about six times the values we obtained for hydra symbionts. Some of these differences could be due to culture and incubation conditions: for example, Pardy and White (1977) showed that fed green hydras have respiration rates threefold higher than unfed controls.

Determinations of photosynthetic O_2 evolution as a function of incident light intensity have been reported for corals and their zooxanthellae (Wethey and Porter, 1976; Crossland and Barnes, 1977). Wethey and Porter (1976) used Michaelis-Menten kinetics to analyze their light intensity-photosynthesis data, claiming that the "K_m" (substrate concentration at half maximal rates of an enzymatic reaction) they obtained corresponded to the "affinity" of photosynthesizing organisms for light.

We analyzed our photosynthetic light response curves on the basis of the maximum rate of photosynthesis, P_{max}; the light intensity at the onset of P_{max}, I_k (Talling, 1957; Yentsch and Lee, 1966), and the initial slope of the light intensity-photosynthesis curve. Changes in the initial slope may be related to changes in the rate of the light reactions at a nonsaturating light intensity, while changes in P_{max} may be ascribed to changes in the overall rate of the CO_2 fixing reactions of photosynthesis (Yentsch and Lee, 1966).

Our data show that symbiont photosynthesis is profoundly affected by both the association with the animal host and the O_2 concentration in the medium. Removing symbionts from their hosts decreases the slope in the light-limited region of the light response curve, which is relatively independent of the O_2 concentration. This effect does not seem to be due to a difference in light-harvesting ability of suspensions of algae and intact hydra due to mutual shading. If this were the case, the more dilute cell suspension should exhibit a steeper slope than that of symbionts *in situ*, as the symbionts *in vitro* are less densely concentrated in the electrode chamber than when they are within the hydra. However, symbionts *in situ* exhibit the steeper slope.

As the oxygen concentration increases from low to near ambient, both the P_{max} and the I_k of symbionts *in vitro* decrease. Such changes have been taken as indicative of a photosynthetic response to an inferior environment (Yentsch and Lee, 1966). In this case the inferior environment is the more highly oxygenated one. In contrast, P_{max} and I_k of symbionts *in situ* change very little or not at all between low and near ambient O_2 concentrations, indicating no change in photosynthetic response over the range of O_2 concentrations examined.

The lower P_{max} of symbionts *in vitro* than that *in situ* indicates inhibition of the CO_2-fixing reactions of photosynthesis. Although this decrease is evident at both O_2 concentrations examined, it is greatest at the higher one. This implies that oxygen (and to a lesser extent the removal of the symbionts from their hosts), acting directly or indirectly on the CO_2-fixing reactions of photosynthesis, inhibits O_2 evolution at light saturation.

Oxygen may inhibit photosynthetic O_2 evolution due to photooxidative damage of the light-harvesting and photosystem pigments, or to pseudocyclic electron flow. Both of these mechanisms have been implicated at relatively high O_2 concentrations in *Chlorella pyrenoidosa* (Shelp and Canvin, 1980). Either mechanism might affect the Calvin cycle by decreasing the reducing equivalents available for CO_2 fixation, thus accounting for the decrease in P_{max}. Oxygen also competes with CO_2 at the active site of the carboxylating enzyme, ribulose 1,5-bisphosphate (RuBP) carboxylase/oxygenase, resulting in the oxygenase function of the enzyme (Chollet and Ogren, 1975; Tolbert, 1979). This reaction produces glycolate, the substrate of photorespiration in higher plants and algae (Goldsworthy, 1970; Chollet and Ogren, 1975; Beck, 1979; Tolbert, 1979). In algae, oxygen reacts at two loci in the photorespiratory pathway: at the RuBP carboxylate/oxygenase and in the conversion of glycine to serine. Hence, the apparent rate of photosynthetic oxygen evolution by algae is reduced with increasing O_2 concentrations. However, at the HCO_3^- concentrations we employed this probably would be minor.

The inhibiting effects of O_2 were markedly less in those algae associated with the host than those *in vitro*. The higher P_{max} in symbionts *in situ* than in symbionts *in vitro* shows that at near ambient O_2 concentrations the maximum photosynthetic capacity of symbionts *in vitro* was drastically less than that of symbionts *in situ*. Furthermore, symbionts *in vitro* were more inhibited by O_2 than those *in situ* when photosynthetic O_2 evolution was examined over a wide range of O_2 concentrations and at light intensities which negated the effects of isolation from the host.

Based on data collected so far a model may be postulated: the close proximity between host and symbiont may lead to a cycling of carbon and O_2 that favors symbiont photosynthesis. Possibly the hydra mitochondrial CO_2 production raises the CO_2 concentration near the algal symbionts, as has been postulated to occur in *Paramecium* (Reisser, 1980). Moreover, host mitochondrial activity can lower the O_2 concentration near the symbionts, which would decrease the inhibitory effects of O_2.

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DISTRIBUTION OF THE ENDOSYMBIONT *NEPHROMYCES* GIARD WITHIN THE ASCIDIAN FAMILY MOLGULIDAE

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ABSTRACT

In various anecdotal reports, nineteenth and early twentieth century authors have asserted that microbial cells, "*Nephromyces*," are present in the renal sac of the ascidian *Molgula*. This study confirms the presence of such cells in the renal sac lumen of five *Molgula* species (*M. manhattensis, M. arenata, M. complanata, M. citrina, M. occidentalis*) and one species of the molgulid genus *Bostrichobranchus* (*B. pilularis*). This is the first report (using modern taxonomic schemes) of *Nephromyces* from a molgulid genus other than *Molgula*.

A description of the light microscope morphology of *Nephromyces* cells is also given.

INTRODUCTION

Like many structures, the "renal sac" of molgulid tunicates was named before critical demonstration of its function. Although it has often been hypothesized (or assumed) that the renal sac is an excretory organ, the biological role of this organ remains uncertain.

Recent work has focused on the morphological and chemical peculiarities of the renal sac. Most unexpectedly for an "excretory" organ, the renal sac has no openings at any stage in its development (Saffo, 1978). Consequently, it has been assumed that renal sac "waste" products are not excreted from the renal sac, but accumulated in the organ for the life of the tunicate (Das, 1948).

The renal sac lumen contains a large volume of concretions, which in composition (chiefly uric acid and calcium oxalate in *Molgula manhattensis*: Goodbody, 1957, 1965; Saffo, 1977a, b; Saffo and Lowenstam, 1978) and possible metabolic origin (Nolfi, 1970) resemble human kidney stones. Unlike kidney stones, however, these concretions show no evidence of being pathological deposits, but seem to be normal metabolic products. The chief organic component of the renal sac fluid in *M. manhattensis* has been identified as homarine (Gasteiger *et al.,* 1960; Saffo, 1976, 1977, Gaill and Lafont, 1978), a methylpyridine. It has been suggested by several authors that homarine is associated with osmoregulation, both in the renal sac of *M. manhattensis* and in the many other marine invertebrates in which the compound has been found (Gasteiger *et al.,* 1960; Lapan, 1975).

At least one feature of the renal sac—the cellular content of its lumen— has been virtually ignored in recent studies. Early papers (de Lacaze-Duthiers, 1874; Giard, 1888; Harant, 1931; Azéma, 1937) assert that fungus-like microbial cells, "*Nephromyces*" (Giard, 1888), are present in the renal sac. Despite their potential

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significance in the activities and biological role of the renal sac, these cells have received little critical attention.

A century after these early reports, even the identity of *Nephromyces* remains in doubt. Usually assuming that these cells represent a single organism, earlier workers have alternately classified *Nephromyces* as a chytridiomycete (Giard, 1888; Harant, 1931), a gregarine protozoan (de Lacaze-Duthiers, 1874), and as "a lower fungus which has no relatives, not even distant ones, among [other groups of lower fungi]" (Buchner, 1965). The cells of *Nephromyces* are so peculiar, their habitat (the renal sac) so bizarre, and published reports so scanty that several recent authors (Johnson and Sparrow, 1961; Alderman, 1976) have questioned the existence of *Nephromyces*, leading Alderman (1976) to state that "*Nephromyces* Giard must be regarded as extremely doubtful unless new evidence becomes available."

This paper confirms the presence of fungus-like cells in the renal sac lumen. A description of the light-microscope morphology of *Nephromyces* is presented, with a report of the distribution of these cells in six species of molgulid tunicates.

MATERIALS AND METHODS

Collection of animals

Molgula manhattensis was collected from the following locations: San Francisco Bay, California (1972–1978, all times of year; Redwood City; Palo Alto Yacht Harbor, Berkeley Municipal Marina, Sausalito Yacht Harbor); Vineyard Sound and Cape Cod Bay, Massachusetts (summers 1977, 1979, 1980; Falmouth, Woods Hole, Vineyard Haven, Sandwich); Chesapeake Bay (September 1977; Solomons Island, Maryland; Gloucester Point, Virginia); Atlantic Coast of Florida (September 1977; Fort Pierce Inlet; Banana River); New Jersey (fall, spring, 1978–1980; Belmar Marina); and Manhattan Island, New York (October 1980; 25th Street Marina).

Molgula citrina and *Molgula complanata* were collected from Sandwich, Massachusetts (summers 1977, 1979, 1980).

Molgula arenata was dredged from Vineyard Sound, Massachusetts (summers, 1977, 1979).

Molgula occidentalis was collected from both the Atlantic (September 1977; Sebastian Inlet) and Gulf Coasts (February 1978 and December–February 1980–1981; Alligator Harbor, Panacea; Carabelle) of Florida.

Bostrichobranchus pilularis was dredged from Vineyard Sound, Massachusetts (summers 1979, 1980; 50 meters depth) and also collected from shallow water in Panacea, Florida, (February 1981; Alligator Harbor).

The *B. pilularis* and *M. occidentalis* from Alligator Harbor were supplied by the Gulf Specimen Co. (Panacea, Florida).

Examination for Nephromyces

Observations were based exclusively on living material. For small animals, the transparent renal sac was excised from the tunicate and examined, whole, with phase contrast optics at 200–400×. For larger animals, the renal sac was dissected from the tunicate, adjoining heart tissue was cut away, and the organ rinsed and blotted with filter paper to remove cells extraneous to the renal sac (*e.g.*, blood cells). Renal sac contents were then removed, placed on a slide, and examined with phase contrast or Nomarski optics at 200–1000×.

TABLE I

Presence of Nephromyces *in the renal sac of adult molgulids, 1977–1981.*

Species	Location	Number examined	Number infected
Bostrichobranchus pilularis	Gulf Coast (Florida) and Atlantic Coast (Massachusetts)	23/23	
Molgula arenata	Atlantic (Massachusetts)	23/23	
M. citrina	Atlantic (Massachusetts)	21/21	
M. complanata	Atlantic (Massachusetts)	22/22	
M. manhattensis	Pacific (California) and Atlantic (Florida to Massachusetts)	212/212	
M. occidentalis	Atlantic and Gulf Coast (Florida)	30/30	

RESULTS

In all adults of all molgulid species examined—*Molgula manhattensis, M. citrina, M. complanata, M. arenata, M. occidentalis,* and *Bostrichobranchus pilularis*—cells are present in the renal sac lumen (Table 1). These cells differ markedly in morphology from tunicate cells, and at least broadly resemble the *Nephromyces* described by earlier authors.

These *Nephromyces* forms can be divided into at least seven broad categories:

1. "Vacuolate filaments": colorless, non-septate filaments, each with a single, large, central vacuole; about 4–10 μm in width, 15 to about 100 μm in length (Fig. 2). Some possess terminal swellings; others are occasionally found in multifilament arrays (Fig. 3). The shortest vacuolate cells are, in some species (*e.g., M. citrina*), sometimes found packaged within a circular structure (Fig. 4). The cytoplasm of vacuolate filaments sometimes contains yellow refractile granular inclusions.

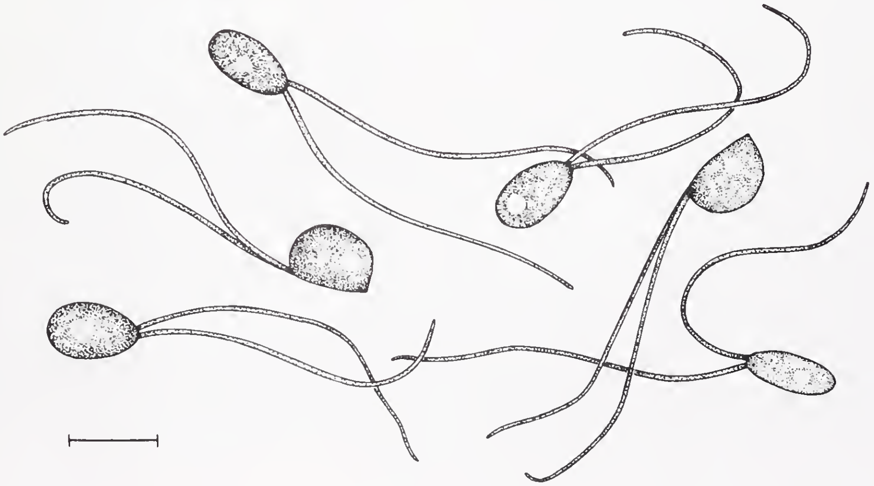
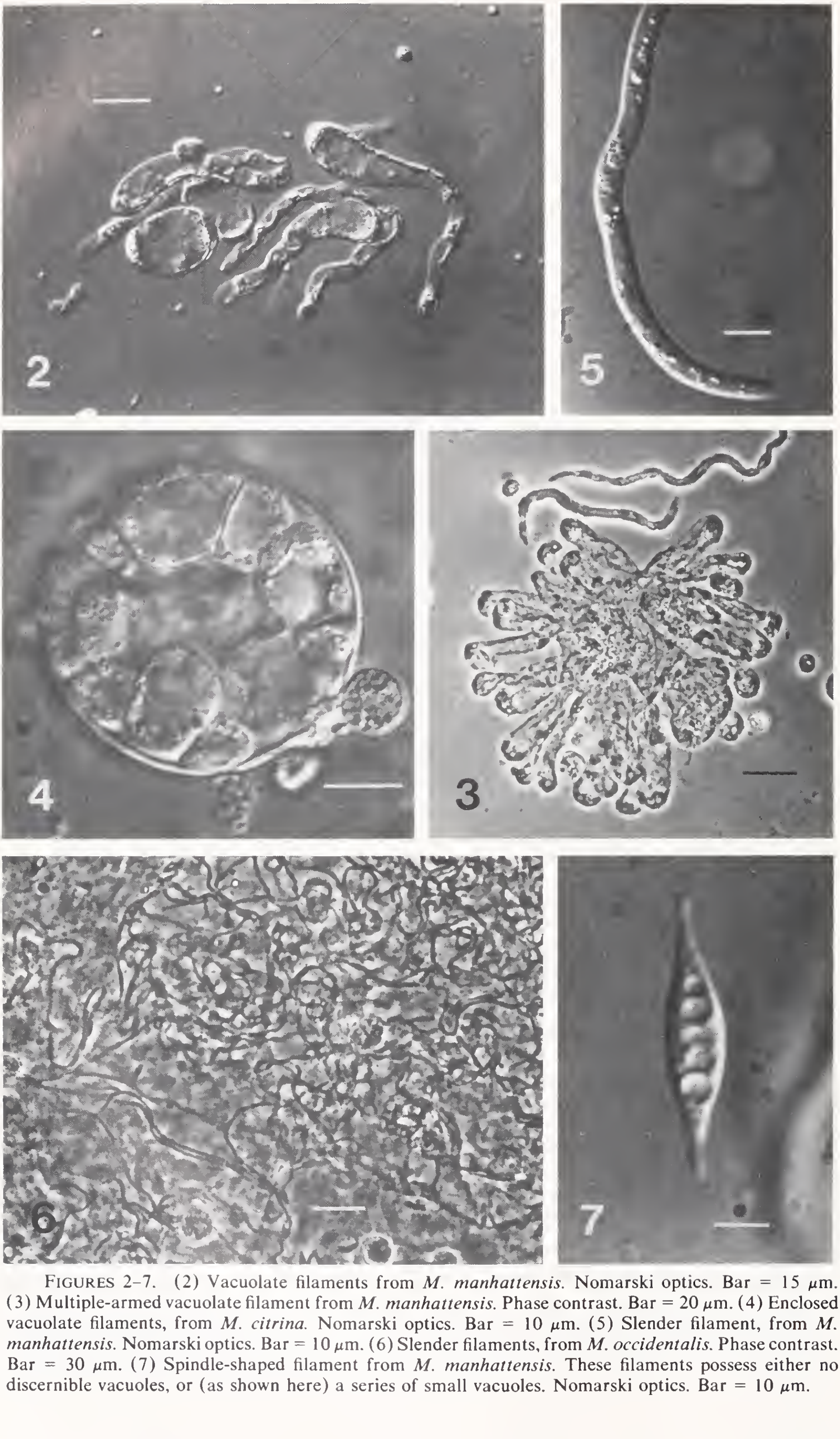


FIGURE 1. Diagrammatic representation of *Nephromyces* zoospores from *Molgula manhattensis.* Bar = 5 μm.

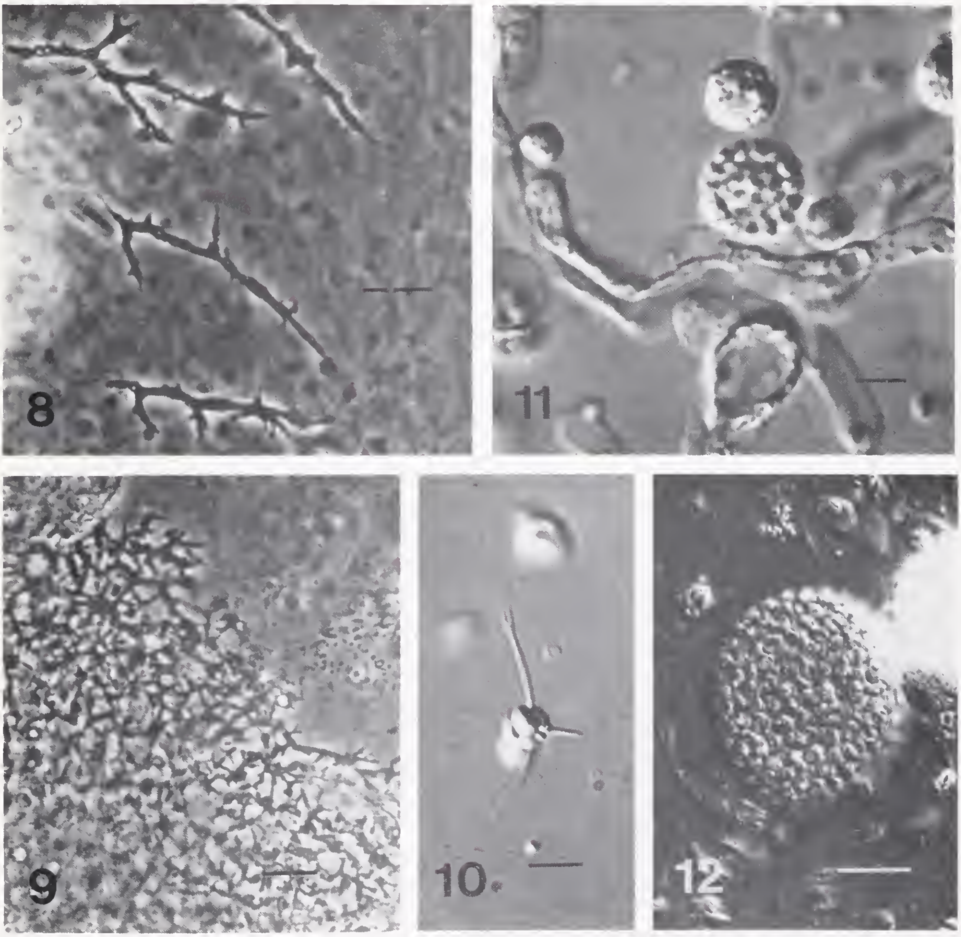


FIGURES 2–7. (2) Vacuolate filaments from *M. manhattensis.* Nomarski optics. Bar = 15 μm. (3) Multiple-armed vacuolate filament from *M. manhattensis.* Phase contrast. Bar = 20 μm. (4) Enclosed vacuolate filaments, from *M. citrina.* Nomarski optics. Bar = 10 μm. (5) Slender filament, from *M. manhattensis.* Nomarski optics. Bar = 10 μm. (6) Slender filaments, from *M. occidentalis.* Phase contrast. Bar = 30 μm. (7) Spindle-shaped filament from *M. manhattensis.* These filaments possess either no discernible vacuoles, or (as shown here) a series of small vacuoles. Nomarski optics. Bar = 10 μm.

2. "Slender filaments": colorless, non-septate filaments without large vacuoles (though occasionally with small vacuoles); 3–5 μm in width, greater than 15 μm in length (Fig. 5); occasionally, some have terminal swellings. In *M. occidentalis* (Fig. 6), these filaments can approach 400–500 μm in length. Filaments usually have rounded tips (Figs. 5, 6); sometimes, though rarely, these filaments have pointed narrow tips (Fig. 7), giving the filaments a spindle-like form.

Both vacuolate and slender filaments are "mycelial" only in the sense that they are often entangled with each other, or with other *Nephromyces* forms; they are virtually never branched, except in early growth stages (Saffo, 1982), and fusion with other filaments has so far not been observed. Occasionally, filaments with characteristics intermediate between those of vacuolate and slender filaments (slender width, but with a row of conspicuous vacuoles separated by thin bands of cytoplasm) are present, suggesting that vacuolate filaments may develop from slender filaments, or *vice versa*.

3. "Irregular filaments": colorless filaments with irregularly shaped bound-

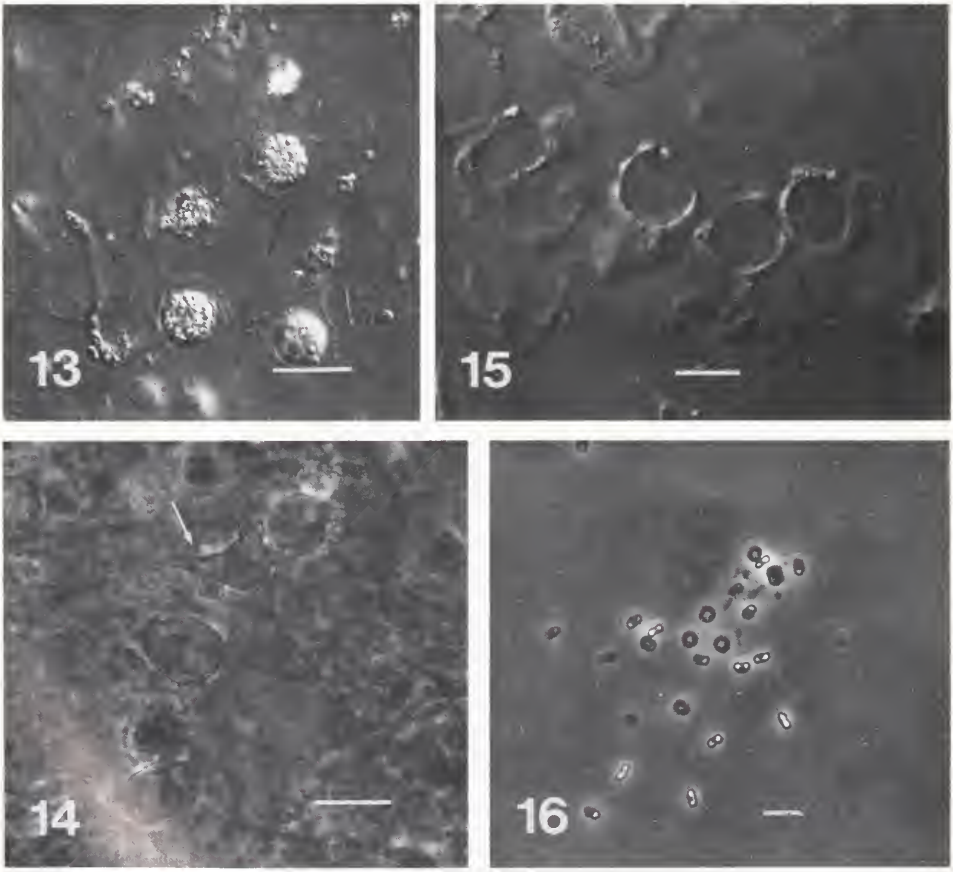


FIGURES 8–12. Irregular filaments on the inner renal sac wall of *M. manhattensis*. Phase contrast. Bar = 15 μm. (9) Network of irregular filaments from *Bostrichobranchus pilularis*. Phase contrast. *Bar* = μm. (10) A zoospore with atypically prominent apical projection, from *M. citrina*. Nomarski optics. Bar = 5 μm. (11) Sporangium (and vacuolate filaments) from *M. manhattensis*. Nomarski optics. Bar = 10 μm. (12) Atypically large sporangium from *M. complanata*. Nomarski optics. Bar = 15 μm.

aries—individually about 3–6 μm in width, typically about 40–60 μm in length (Fig. 8). Unlike other filament forms, the irregular filaments are found more often on the inner wall of the renal sac, rather than free in the renal sac lumen. They are sometimes found in network-like arrays (Fig. 9). To date, such filaments have been found only in *M. manhattensis* and *B. pilularis.*

4. Posteriorly biflagellate swarmer cells ("zoospores"): the cell is about 3–5 μm in length, with one or two refractile globules in the center of the cell. The flagella are equal in length, about 12–15 μm long (Fig. 1). In some species (*e.g., M. citrina*), zoospores occasionally possess a prominent apical projection (Fig. 10).

5. Rosette-shaped "sporangia": usually about 20 μm diameter (Fig. 11), though sometimes larger (an occasional occurrence in *M. complanata* and *M. citrina* Fig. 12). Biflagellate zoospores (above) are discharged from these. Before zoospores discharge, the sporangium is surrounded by a wall, which is apparently dissolved or torn apart during zoospore discharge. No discharge pore has been detected in the sporangium in most host species, although in *M. citrina*, sporangia are often surrounded by a heavy wall with two or three tunnel-like openings (Fig. 13); some-



FIGURES 13–16. (13) Sporangia surrounded by heavy tunneled wall (arrow), from *M. citrina.* Nomarski optics. Bar = 20 μm. (14) Sporangial wall connected to filament (arrow), from *M. citrina.* Phase contrast. Bar = 15 μm. (15) "Baskets" from *M. manhattensis.* Nomarski optics. Bar = 10 μm. (16) "Doughnut"-shaped cells from *M. manhattensis.* Phase contrast. Bar = 10 μm.

times these walled structures seem to be connected to filaments (Fig. 14). Yellowish spherical granular cells, about 20 μm in diameter, are also present; their size and form suggest that they are uncleaved sporangia.

6. "Baskets": irregularly shaped structures (Fig. 15), open at one end (prominent rings in Fig. 15), and virtually devoid of cytoplasm; about 25–50 μm in diameter. Though it is not clear whether these structures are even living cells, they may represent remnants of some *Nephromyces* cell type. Certainly they are associated only with *Nephromyces.* I have never seen them anywhere in *Molgula* other than in the renal sac, and they do not appear in experimentally *Nephromyces*-free *Molgula* (Saffo and Davis, 1982).

7. "Doughnuts": heavy-walled flattened circular cells, about 3–5 μm in frontal diameter (Fig. 16). The behavior of these cells will be described more fully elsewhere (Saffo, unpublished).

Except for the irregular filaments (3, above), all these cell types were found in the six molgulid species examined. All these cells were usually (but not invariably) found in each adult of each species, though relative numbers of each cell type often varied from individual to individual. In contrast to Giard (1888) and Harant (1931), I have seen no qualitative differences in cell-type distribution with season, at least in adult *M. manhattensis* (the only species sampled at all times of year).

DISCUSSION

Although these observations are broadly similar to those of earlier authors, their descriptions do differ from mine—and from each other—in many details, where they provide these at all.

De Lacaze-Duthiers (1874) stated that filaments (reminiscent of types 1 and 2 above), were "almost always present" in *M. tubulosa* (=*M. occulta*: Berrill, 1950), but he saw no other forms.

Giard found "*Nephromyces molgularum*" in *M. socialis* (=*M. manhattensis*: Berrill, 1950); "*N. sorokini*" in *Lithonephrya eugyranda* (= *M. complanata*: van Name, 1945) and "*N. roscovitanus*" in *Anurella roscovitana* (=*M. occulta*: Berrill, 1950). Of these, he described only *Nephromyces molgularum* in any detail and provided no illustrations. This species, according to Giard, possesses a mycelium of delicate, entangled filaments, some of which have terminal swellings. Zoosporangia of "very diverse form" liberate tiny, uniflagellate zoospores with a refringent granule near the base of the flagellum. In autumn, zygospores are formed where "four or five" mycelial filaments fuse; in winter, filaments germinate from these zygospores. Vacuolated filaments are present all year long.

Harant (1931) found filaments of various lengths, with and without vacuoles, in the molgulid *Ctenicella appendiculata* (=*Molgula appendiculata*: Buchner, 1930). Harant also described filaments which bear spiny-surfaced "resistant spores," which he considered markedly different from the zygospores of Giard. He reported that cylindrical, vacuolated cells develop into zoosporangia; these release "zoospores" with a lipid globule and single, long, apical flagellum. These "zoospores" (belying the asexual implications of their name) fuse with each other as gametes.

Of all the earlier reports of *Nephromyces*, Buchner's (1930, 1965) descriptions of *Nephromyces* from *M. impura* most nearly resemble those presented here—at least in description of the morphology of *Nephromyces* cells, if not in interpretation of their developmental roles. Buchner (1965) also noted that he found "more or less similar forms (of fungi) in a long series of molgulids, preserved in alcohol, from all parts of the earth."

At the light microscope level, each of the cell types described here, as in earlier papers, bears a superficial resemblance to very different kinds of microorganisms. The non-septate filaments (types 1 and 2) resemble phycomycetous fungi. The irregularly-bounded filaments seem more similar to slime molds (and associated mycetozoans) than to fungi. The sporangia strongly resemble those of thraustochytrids (*e.g.*, Goldstein, 1963) in behavior and in light microscope structure; but the swarmers that are discharged from them are markedly different from those of thraustochytrids, even at the light microscope level. The swarmer cells do resemble chytrid zoospores in size, in the presence of refractile globules, and in their posterior flagellation. However, chytrid zoospores are typically uniflagellate rather than biflagellate; in fact, there is no flagellate protistan group with posteriorly biflagellate zoospores (Saffo, 1981). At the light microscope level, the apical projection in some *Nephromyces* swarmers (Fig. 10) broadly resembles a short or rudimentary variant of the haptonema borne on flagellated cells of the algal haptophytes (Prymnesiophyceae), except that the *Nephromyces* projection does not appear to be motile. Finally, in their small size and simplicity of surface (light microscope) morphology, the doughnut-shaped cells resemble prokaryote cells more than they do eukaryotes.

The appearance of *Nephromyces* raises interesting phylogenetic questions (Saffo, 1981), to which ultrastructural data will contribute essential portions of the answers. Meanwhile, two basic questions must be addressed.

If *Nephromyces* cells resemble so many different kinds of organisms, is *Nephromyces* one kind of organism, or a collection of several kinds of organisms? Though these observations do not prove that *Nephromyces* is (within each host species) a single organism, they are consistent with this hypothesis. In the six molgulid species studied, the same categories of *Nephromyces* cells appear repeatedly, with only slight variations from host species to host species. If *Nephromyces* were merely a group of unrelated organisms inhabiting the renal sac, it would be difficult to imagine the same (or similar) community persisting in species after species of molgulids, despite wide differences in (1) morphology, (2) habitat (floats and pilings, *M. manhattensis* and *M. citrina*; sandy bottom, *M. arenata*; intertidal rocks, *M. complanata*; mud and sandy mud, *B. pilularis*), and (3) developmental pattern (oviparous, *M. manhattensis, M. occidentalis, M. arenata*; viviparous, *M. citrina, M. complanata*; oviparous with direct development, *B. pilularis*) of the host species in question. More substantial developmental evidence in support of this hypothesis is presented elsewhere (Saffo, 1981, 1982). The only occasional presence of irregular filaments is not inconsistent with this hypothesis, since irregular filaments are almost certainly an early or alternate developmental stage of the slender filaments (Saffo, unpublished).

Slight variations in *Nephromyces* are found in different host species. Such differences persisted even in the single case where two molgulids (*M. manhattensis* and *M. citrina*) cohabited the same area (Sandwich Marina). Either different host species do possess different *Nephromyces* species, as Giard suggested (1888), or the renal sacs in different host species are sufficiently different habitats to induce developmental differences in a single *Nephromyces* species.

Is *Nephromyces* an organism at all, or merely molgulid cells? If one accepts the older reports, it can be concluded that *Nephromyces* is present in molgulids not only from the western Atlantic and eastern Pacific, but also from the eastern Atlantic and Mediterranean, and in three molgulid species in addition to the six enumerated in this paper. At the very least, the distribution of *Nephromyces* within the Molgulidae is taxonomically and geographically widespread. The presence of *Nephromyces* in *Bostrichobranchus pilularis* is particularly striking, as it is the first report (using current taxonomic schemes) of *Nephromyces* in a molgulid other than the genus *Molgula*. Indeed, these results suggest that its distribution may well be universal among adult molgulids. I have not found any adult molgulid specimen that does not contain *Nephromyces* in its renal sac.

The possibly universal distribution of *Nephromyces* leads one to question whether *Nephromyces* is a microorganism or merely a collection of molgulid cells. At the light microscope level, *Nephromyces* cells do not look like tunicate cells. A subsequent paper (Saffo and Davis, 1982) presents critical evidence that *Nephromyces* is, indeed, something foreign to its host.

If *Nephromyces* is not merely a collection of molgulid cells, its universal distribution among the molgulid species studied, and, by implication, widespread distribution throughout the Molgulidae make it difficult to imagine that *Nephromyces* has grossly pathologic effects on its hosts. Though it has not been demonstrated that the association between molgulids and *Nephromyces* is mutualistic, it does seem to be an intimately coevolved association. Certainly, investigation of this association appears to be essential for understanding of both the role of the renal sac, and, more generally, of the evolution and ecology of the common, but poorly understood Molgulidae.

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MODES OF INFECTION OF THE ASCIDIAN *MOLGULA MANHATTENSIS* BY ITS ENDOSYMBIONT *NEPHROMYCES* GIARD

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ABSTRACT

The renal sac of the sea squirt *Molgula manhattensis* consistently harbors a collection of fungus-like cells, "*Nephromyces*". These cells are not *Molgula* cells, but an organism(s) foreign to the host. *Nephromyces* does not have an obligate intermediate host. *Nephromyces* is not transmitted with the gametes of *Molgula*, but can be transmitted to *Molgula* through the ambient water. *Nephromyces* is released into the water after death of its host, although not necessarily only at this time. *Molgula* acquires *Nephromyces* after the initiation of feeding, which follows settling and metamorphosis. *Nephromyces* remains infective for at least twenty-nine days after isolation from its host.

INTRODUCTION

In the renal sac of all adults of all molgulid tunicate species so far examined (Saffo, 1982), fungal-like cells, *Nephromyces* Giard (1888), are present in conspicuous numbers.

The ubiquity of *Nephromyces* cells in the renal sac of these molgulids suggests that the interaction between *Nephromyces* and molgulids may be a symbiotic association with important effects on the activities of the renal sac and on the general biology of molgulids. To consider physiological and ecological aspects of such questions, it would be useful to understand the developmental relations between *Nephromyces* and its hosts. Are *Nephromyces* cells and molgulid tunicates associated with each other throughout the life cycles of both host and endosymbiont? How is *Nephromyces* transferred from its ductless habitat, the renal sac, to a new host? How is this association maintained, generation after generation?

The universality of the association between *Nephromyces* and at least six molgulid species also raises the most important question: is this really a symbiotic association, or is "*Nephromyces*" merely a collection of molgulid cells?

In considering some of the developmental interactions between *Nephromyces* and the ascidian *Molgula manhattensis*, this paper presents critical evidence for the existence of *Nephromyces*, and describes some means by which *Nephromyces* cells from *M. manhattensis* are transferred from host to host.

MATERIALS AND METHODS

We collected adult *Molgula manhattensis* from San Francisco Bay, California and Vineyard Sound, Massachusetts.

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Nephromyces-*free* Molgula (*experiments* 1–5)

In the laboratory, field-collected adults were rinsed of debris and ambient water, then induced to spawn into 0.22 μm-filtered seawater (Saffo, 1978). Embryos were maintained in 0.22 μm-filtered seawater through metamorphosis and initiation of feeding by the settled zooids (Fig. 1). California-raised *M. manhattensis* were grown beyond this stage of development in coarsely-filtered (filtrate with particle size ≤ 1 mm) seawater collected from Bodega Bay, California, where *Molgula* (and *Nephromyces*) are not present. Massachusetts-raised *M. manhattensis* were grown in 0.8 μm-filtered seawater. These laboratory-grown *M. manhattensis* were fed a variety of axenically cultured unicellular algae, including *Dunaliella, Platymonas, Isochrysis, Monochrysis, Stichococcus*, and *Nannochlorus* spp.

Inoculation experiments

For experiments 1–4, laboratory-raised, *Nephromyces*-free *M. manhattensis* zooids were inoculated with *Nephromyces* by introduction of renal sac contents (including renal sac fluid, and some concretions, as well as *Nephromyces* cells) freshly dissected from sexually mature, field-collected *M. manhattensis*, into the ambient water of the settled zooids. After varying periods of exposure to *Nephromyces* cells (1 hour to 9 days at 16–19°C), culture dishes on which zooids had settled were rinsed thoroughly with 0.22 μm-filtered seawater.

In experiment 3, other modes of inoculation were tested as well. Some laboratory-raised, *Nephromyces*-free zooids were incubated at 17°C with living, *Nephromyces*-infected *M. manhattensis* (7–9 days in a one gallon aquarium with one or two field-collected adults; 54 days in a five gallon aquarium with 20 laboratory-

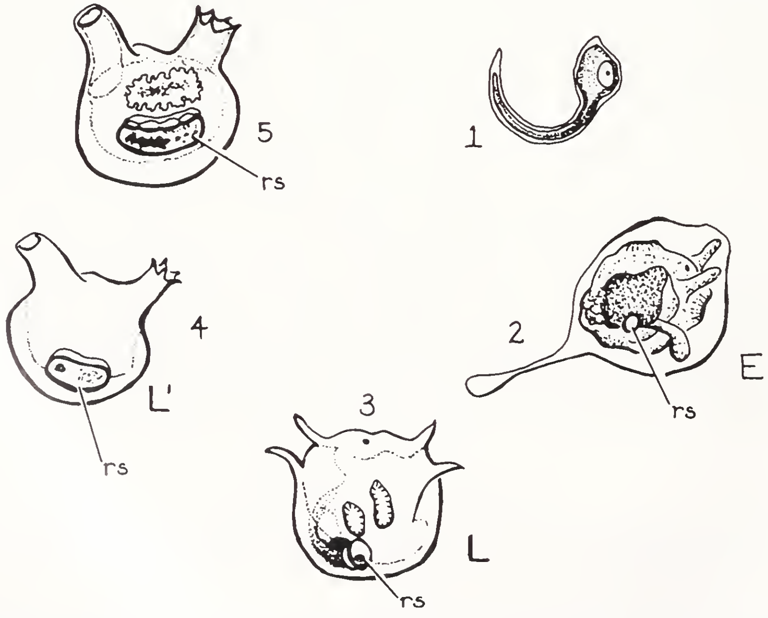

FIGURE 1. Inoculation of *Molgula* with *Nephromyces* before ("early": E) and after ("late": L, L') initiation of feeding. 1: tadpole larva; 2: settled, metamorphosing zooid (renal sac present, feeding organs not functional); 3: feeding zooid (5–7 days after fertilization); 4: zooid several months after fertilization; 5: sexually mature adult. *rs*, renal sac. Drawings not to scale.

raised zooids). Other *Nephromyces*-free zooids were settled onto slides, marked and introduced into San Francisco Bay in a crab trap one meter below the underside of a float in the Berkeley municipal marina. Large aggregations of (field-settled) *M. manhattensis* were present on this float. One group of such laboratory-settled zooids was suspended in the bay for five days in April 1978 (water temperature: 18°C). A second group was suspended in the bay for 13 days in July 1978 (water temperature: 20°C).

Each set of inoculation experiments included an uninoculated, control culture of laboratory-raised *M. manhattensis.*

Persistence of infectivity of Nephromyces *cells in seawater* (experiment 5)

Freshly isolated *Nephromyces* cells were centrifuged (20 min, 4°C, 900 *g.*) and, after decanting the renal sac fluid supernatant, resuspended in autoclaved, 0.22 μm-filtered seawater containing penicillin and streptomycin (0.25 g/l each). Such cell suspensions were added as inocula to cultures of *M. manhattensis* (feeding zooids) one, two, four and twenty-nine days after suspension in seawater, and rinsed from *M. manhattensis* cultures after eight hours' incubation at 19°C, or twenty-four hours' incubation at 16°C. A seven-day-old seawater suspension of such *Nephromyces* was filtered through a 5 μm Nuclepore filter, the filtrate then incubated with a *Nephromyces*-free *M. manhattensis* culture for 24 hours at 19°C.

After twelve or more days, *Nephromyces*-treated and control cultures of *M. manhattensis* were checked for the presence of *Nephromyces* in the renal sac lumen by examination of the transparent renal sac with phase contrast optics at 200–400×.

RESULTS AND DISCUSSION

Experiment 1: Are *Nephromyces* cells passed on to the next generation of *Molgula manhattensis* with the gametes of the adult oviparous ascidian? Of the *M. manhattensis* raised from naturally-spawned eggs and sperm in *Nephromyces*-free seawater (ten separate experiments), none of the 266 zooids examined were infected with *Nephromyces*. Thus, *Nephromyces* is not transmitted with the sperm or eggs of *Molgula manhattensis.*

Field observations are consistent with these laboratory data. Although all adult *Molgula manhattensis* are infected with *Nephromyces* (Saffo, 1982), the renal sacs of immature *M. manhattensis* are not invariably infected (Table I). Further,

TABLE I

Percent Nephromyces-*infected individuals among field-settled* Molgula *zooids (San Francisco Bay)*

Collection date	Sexually mature?	Size (mm)	N	% Infected	Smallest infected individuals (mm)	Minimum size (mm) 100% infection
I. 5/15/78	no	≤2 (0.3–2.0)	10	0	3.5	≥13
	no	2–6	22	23		
	no	6–10	9	44		
	no (<13 mm); yes (≥13 mm)	10–14	5	80		
	yes	>14 (17–33)	11	100		
II. 6/19/78	no	≤2 (1.5–2.0)	4	25	1.5	≥2.5
	no	2–8	4	100		
	yes	>8 (20–30)	12	100		
III. 8/13/78	no	≤2 (0.8–2.0)	10	100	0.8	≥0.8
	no	>2 (4.0–10.5)	3	100		

the rate of infection of young *M. manhattensis* varies with season. Of 57 *M. manhattensis* (0.3–33.0 mm in diameter) collected from Berkeley marina (San Francisco Bay) early in the reproductive season (May 1978), only the renal sacs of sexually mature animals (diameter > 13 mm) were always infected with *Nephromyces*. No individual smaller than 3.5 mm possessed an infected renal sac. Of 20 *M. manhattensis* (1.5–30 mm in diameter) collected from the same location in June, 1978, all individuals larger than 2 mms were infected. In August, even the smallest zooids collected from San Francisco Bay (0.8 mm) were infected with *Nephromyces*. If *Nephromyces* were transmitted with the gametes of *M. manhattensis*, such seasonal differences in infection rate would not be expected.

Experiment 2: Does *Nephromyces* require passage through a second host before reinfection of *M. manhattensis*? As Tables II, III, and IV show, *Molgula* are infected with *Nephromyces* by addition of these cells to ambient seawater. Although *Nephromyces* may infect organisms other than molgulids, these experiments do indicate that *Nephromyces* does not absolutely require such an alternate host before successful reinfection of *M. manhattensis*.

Experiments 1 and 2: Are *Nephromyces* really *Molgula* cells, or, as their morphology suggests (Saffo, 1982), are they an organism(s) foreign to *Molgula*? If *Nephromyces* cells were merely tunicate cells, one would expect to always see *Nephromyces* cells in the renal sac, no matter how *M. manhattensis* were raised. Instead (experiment 1), *Nephromyces*-free *M. manhattensis* can be obtained routinely. Furthermore, the lumen of an uninfected renal sac is free not only of *Nephromyces* cells, but of *all* cells—making misinterpretation of such observations virtually impossible. This *Nephromyces*-free condition persists in all uninoculated

TABLE II

Modes of transmittance of Nephromyces *from host to uninfected zooids*

Mode inoculation	Control # (this table)	Time of inoculation (days after fertilization)	Period of inoculation (days)[c]	Size of inoculum	Time of examination (mos. after inoculation)	N examined	% Infection
1. — (Control)	—	—	—	—	2	32	0
2. — (Control)	—	—	—	—	4	20	0
3. — (Control)	—	—	—	—	4	21	0
4. — (Control)	—	—	—	—	6	9	0
5. — (Control)	—	—	—	—	9	7	0
6. — (Control)	—	—	—	—	1	26	0
7. Living infected adult	1	53	7	1 (25 mm) animal/l liter	1	19	0
8. Living infected adult	2	2	9	2 (20 mm) animals/3 liters	3, 4	17	0
9. Field (S.F. Bay)	1	4/9/78; 4	5	—	2	26	0
10. Field (S.F. Bay)[b]	5	7/5/78; 0 (field-settled) + 257 (lab-settled)	13	—	1	23 (17 lab/6 field)	100
11. Infected Laboratory Zooids[b]	1	113	54	20 (2 mm) animals/21 liters	2	20	100
12. Renal Sac contents[a,b]	2	2	9	2 renal sac/l liter	2, 4	23	100
13. Renal Sac contents[a,b]	3, 4	173	9	2 renal sac/l liter	6	3	100
14. Renal Sac contents[a,b]	6	6	1	2 renal sac/l liter	1	16	100

[a] See also tables 3 & 4 for shorter inoculation periods.

[b] Inoculum included dead animals (or portions of dissected animals).

[c] Temperature = 17–20°C in all cases.

TABLE III

Percent Nephromyces-*infected individuals among laboratory-raised* Molgula *infected "early" (before siphons open) and "late" (after siphons open) in metamorphosis (Figure 1)*

Trial #	Time of inoc. after fertilization (days)	Period of inoculation (hours)/ temperature	Time of examination after fertilization (days)	N Examined	% Infected
1	C:[a] —	—	24	25	0
	E:[a] 1.5	1/(19°C)	24	27	0
	L:[a] 4	1/(19°C)	24	25	100
2	C: —	—	23	25	0
	E: 2	1/(19°C)	24	28	3.6
	L: 4	1/(19°C)	22	25	100
3	C: —	—	26	25	0
	E: 2	1/(15°C)	31, 36, 94	28	0
	L: 7	1/(15°C)	26–31	27	63

[a] C = control; E = early; L = late.

M. manhattensis, no matter what the general condition of the animals themselves, or the contents (other than absence of *Nephromyces* cells) of ambient water. If *Nephromyces* cells were really tunicate cells that appeared only in certain environmental situations, one would expect to see some exceptions to these results. If, for instance, *Nephromyces* cells were *Molgula* cells which arose only in response to microbial infection, one would expect that the renal sac of *M. manhattensis* raised in coarsely-filtered (but *Nephromyces*-free) seawater, which can contain a variety of protists, fungi and small metazoans, might occasionally be infected with *Nephromyces* cells—but they are not. Likewise, one might also expect unhealthy (clogged intestine, feeble siphon response, infestations of ciliates or bacteria in the tunic), uninoculated *Molgula* to possess *Nephromyces* cells—but they do not. In short, *M. manhattensis* contain *Nephromyces* cells only if the *Molgula* cultures

TABLE IV

Infectivity of Nephromyces *after isolation from the renal sac*

Nephromyces inoculum	Days of axenic incubation of *Nephromyces* after isolation from renal sac	Period of inoculation (hrs.)	Date of inoculation (days after fertilization of M. manhattensis)	Date of examination (days after fertilization/ inoculation	N Examined	% Infection
1[a]—(Control)	—	—	—	24/—	25	0
2[a] +	1	8	32	57/25	25	100
3[a] +	2	8	33	58/24	25	100
4[a] +	4	8	35	58/23	25	100
5[a] +	7	24	38	58/20	25	100
6[b] +	29	24	6	18/12	32	100

[a] inoculation/incubation temperature: 19°C.
[b] Inoculation/incubation temperature: 16°C.

have been specifically inoculated with *Nephromyces* cells. Thus, *Nephromyces* cells are not *Molgula* cells; they are cells foreign to the tunicate.

Experiment 3: How are *Nephromyces* cells transmitted from the ductless renal sac to a new host? Experiment 1 indicates that *Nephromyces* is not released with the gametes of *M. manhattensis* at spawning. Experiment 2 indicates that *M. manhattensis* can be infected by *Nephromyces* released directly into seawater after dissection from the renal sac. In natural conditions, are *Nephromyces* cells released from *Molgula* only in a similar event (death of the host), or are they released from living animals as well? One month after 7–9 days' incubation of laboratory-raised, *Nephromyces*-free *M. manhattensis* zooids with living, field-collected, *Nephromyces*-infected *M. manhattensis*, none of the lab-raised *M. manhattensis* had become infected (Table II). After 54 days' incubation of lab-raised *Nephromyces*-free zooids with lab-raised, *Nephromyces*-infected zooids, all of the formerly *Nephromyces*-free zooids had become infected. *Nephromyces* may be released infrequently, or in small numbers from living *Molgula*, such that only lengthy exposure of the inoculum results in 100% infection of young zooids. Or, since several *Nephromyces*-infected zooids died in the aquarium during the course of the long-term incubation, *Nephromyces* may be released only from dead *Molgula*. Our laboratory experiments do not distinguish between these two alternatives, but the following observations favor the latter possibility.

In early April, a group of lab-raised *Nephromyces*-free zooids was suspended in San Francisco Bay for five days. Extensive aggregations of "wild" *M. manhattensis* (only a few of them sexually mature adults) were located one meter away on adjacent floats. Eight weeks later, none of the lab-raised zooids were infected with *Nephromyces*. In early July, a second group of such lab-raised zooids was suspended in the bay for 13 days, again with extensive aggregations of *M. manhattensis* (this time virtually all of them adults) on neighboring floats, and with several dead *M. manhattensis* floating in the water within a meter of the lab-raised zooids. Three weeks after removal from the bay, all lab-settled *M. manhattensis* (6–10 mm diameter) and young field-settled *M. manhattensis* (1–2 mm diameter) were infected with *Nephromyces*. Although the difference in incubation time in the two field trials may be partly responsible for the differences in infection rates, both the striking difference of rate (0% *vs.* 100%) and the short time required for infection in the laboratory (40–60 minutes, experiment 4) suggest that other factors—especially seasonal differences in numbers and death rate of nearby adult *M. manhattensis*—might be chiefly responsible for the differences in infection rate.

None of these experiments eliminate the possibility that *Nephromyces* cells are released from living *M. manhattensis*. However, in all the successful inoculation trials–and only in these trials—dead infected *M. manhattensis* were clearly part of the potential source of infection. This is consistent (as is our standard laboratory infection procedure) with the possibility that, in nature, *Nephromyces* cells are released into the ambient water only upon death of their tunicate host.

Experiment 4: How does *Nephromyces* enter a new host? *M. manhattensis* exposed to *Nephromyces* at different stages of development—after settling and formation of the renal sac but before the siphons open (Fig. 1, "early inoculation," Saffo, 1978), and after the siphons open (the beginning of feeding, "late inoculation")—show striking differences in infection rate (Table III). In two of the three trials (Table III), none of the *M. manhattensis* inoculated "early" in metamorphosis were infected with *Nephromyces* 3–13 weeks after inoculation. In a third trial, only a single *M. manhattensis* (out of 28 examined) was infected. In contrast, 63–100% of the *M. manhattensis* inoculated after completion of metamorphosis were infected with *Nephromyces* a month after inoculation. This indicates that *Nephromyces* enters *Molgula* most readily through the siphons of the host, presumably most easily through the incurrent siphon, in feeding. While the single infected "early-inoculated" *Molgula* may have become infected by *Nephromyces* in some other manner (*e.g.*, penetration of the tunic), results of experiment 5 (below) are consistent with the hypothesis that *Nephromyces* might occasionally survive post-inoculation rinsing, persist on the surface of *Molgula* or the culture dish, and later be ingested by *Molgula* zooids after they start feeding.

This experiment establishes the developmental stage at which *Molgula* is ingested, and the general way in which *Nephromyces* enters its host. It does not establish, however, how *Nephromyces* enters the ductless renal sac; the latter process will be described elsewhere (Saffo, unpublished).

Experiment 5: *Nephromyces* cells are not transmitted from *Molgula* with gametes of the host. Rather, they are transmitted through the ambient water—perhaps only from dead, ingested *Molgula* and probably to new *Molgula* only via feeding currents. How can such an apparently haphazard mode of transmission lead to 100% infection of adult *M. manhattensis* by *Nephromyces*—especially early in the reproductive season, when the numbers of adult *Molgula* (the ultimate source of *Nephromyces*) are low?

M. manhattensis can be readily infected with *Nephromyces* immediately after initiation of feeding, and also long after completion of metamorphosis (Table II). Laboratory-raised animals were successfully inoculated as long as 9 months after fertilization; the largest such animal (L′, Fig. 1) was 77 mm in diameter, and showed initial traces of gonad formation (gonoduct, immature oocytes, *etc.*). Patterns of infection of *M. manhattensis* in the field (Table I) are consistent with these experimental data. The susceptibility of *Molgula* to infection by *Nephromyces* for a long developmental period after the initiation of feeding must extend the chances for infection.

These chances are also favored because at least some *Nephromyces* cells can retain their infectivity long after isolation from their host. Exposure of *M. manhattensis* zooids to *Nephromyces* incubated one, two, four, seven or twenty-nine days in seawater (Table IV) invariably results in 100% infection of *Molgula*.

This experiment, as well as experiments 1 and 4, indicate that *Molgula* and *Nephromyces* can survive independently for extended periods. Certainly, neither organism is associated with the other throughout its entire life cycle. Are *Molgula* and *Nephromyces* nevertheless dependent on each other for completion of their life cycles? Since the *Nephromyces* cell which survives longest in seawater may be a resistant spore (Saffo, unpublished), the survivability of *Nephromyces* outside *Molgula* is still compatible with the notion that infection of *Molgula* is an obligate portion of *Nephromyces*' life cycle.

The dependence of *Molgula* on *Nephromyces*, however, is more problematic. Since *Molgula* does not acquire *Nephromyces* until after metamorphosis, *Molgula's* dependence (if any) on *Nephromyces* must be in some aspect of post-metamorphic metabolism, growth or development.

ACKNOWLEDGMENTS

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CYTOCHEMISTRY OF THE LONG-NECKED CELLS IN THE FOOT OF *ONCHIDORIS MURICATA* (NUDIBRANCHIA)

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ABSTRACT

The long-necked cells of the foot on *Onchidoris muricata* were found distributed among the shorter secretory cells which composed most of the epidermal tissue. The long-necked cells extend through the basement membrane on which the smaller cells lie. The ultrastructure of the cell body at the base of the cell shows the nucleus, large amounts of rough endoplasmic reticulum, and an extensive Golgi body that produces large numbers of secretory granules. The periodic acid-thiosemicarbazide-silver protein (PA-TSC-SP) test for polysaccharides was negative for the secretory material. These results are consistent with polysaccharides having large obstructing groups such as sulfates. The high iron diamine (HID) test for sulfated carbohydrates was positive in the Golgi saccules and secretory granules at the base of the cell. Tannic acid fixation for glucosaminoglycans was only slightly positive in the extracellular matrix. Alcian blue stained tissue for light microscopy complimented the ultrastructural cytochemical tests and indicated that the secretory materials are sulfated mucopolysaccharides.

INTRODUCTION

Although the ultrastructure of secretory cells producing various carbohydrate complexes has been thoroughly studied in mammalian systems, particularly in tissues specialized for fairly narrow functions, similar studies in gastropod secretory tissue are rare (Ovtracht, 1967; Ovtracht, *et al.*, 1969; Storch and Welsch, 1972; Chailley, 1979). Ultrastructural studies of multifunction secretory tissues in gastropods are even less common (Porter and Rivera, 1980).

The chemical components of mucoid materials secreted by various types of cells have been studied at the ultrastructural level by autoradiographic methods (Neutra and Leblond, 1966) and chemical stains (Thiéry, 1967; Friend, 1969; Spicer, *et al.*, 1978). Many cells containing secretory granules in epidermis of some nudibranchs are periodic acid Schiffs positive at the light optical level (Porter and Rivera, 1980). At the ultrastructural level, Porter and Rivera (1979) have demonstrated periodic acid oxidizable carbohydrates in the secretory granules of ceras epidermal cells of *Aeolidia papillosa.*

In this study, the large, secretory, long-necked cells of the foot of the nudibranch *Onchidoris muricata* were studied for their ultrastructural morphology and chemical constituents of the secretory granules. The limited distribution of these cells at the anterior of the foot generated an interest to determine the composition and function of long cell secretory product. The chemical analyses were primarily aimed at differentiating mucoid materials used for locomotion and adhesives for attachment to substrates (Cranfield, 1973a, 1973b).

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Abbreviations: PA-TSC-SP, periodic acid-thiosemicarbazide-silver proteinate; HID, high iron diamine; PAS, periodic acid-Schiffs.

MATERIALS AND METHODS

Nudibranch tissue

Onchidoris muricata adults were collected from under rocks at low tide from King's Beach in Lynn, Massachusetts, and Rye Beach, New Hampshire, in the autumn of 1979. Whole animals were fixed with 5% glutaraldehyde in 0.075 *M* NaOH-PIPES (Piperazine-N,N′ bis [2-ethane sulfonic acid]) buffer pH 7.4 overnight at 4°C except for specimens used for extracellular matrix detection as noted below. The foot anterior was isolated and processed for light and electron microscopy.

Electron microscopy

Small tissue blocks (2–3 mm on a side) of fixed foot anterior were stabilized at pH 6.8 in 0.075 *M* NaOH-PIPES buffer and then post stained in 2% osmium tetroxide in the same buffer for 1.5 h. The tissue was then dehydrated in an alcohol series and embedded in Spurr's plastic (Spurr, 1969). Ultrathin sections were obtained with a Porter-Blum Ultra-microtome MT-1. Observations and micrography were made with a Zeiss EM 9S-2 transmission electron microscope operated at 60 KV. All ultrathin sections were mounted on gold grids for all ultrastructural observations and electron microscopic cytochemical tests.

Cytochemical stains—Tests for periodate oxidizable carbohydrates were done as described by Thiéry (1967). In this procedure sections were oxidized for 30 min in periodic acid (PA). Thiosemicarbazone derivatization of oxidized carbohydrate was carried out with thiosemicarbazide (TSC) for 72 h. Silver was deposited at these sites after exposure of the sections to silver proteinate (SP) for 30 min. Appropriate controls leaving one or more reagents out of the reaction were employed to check the specificity of this test (Porter and Rivera, 1979).

For detection of sulfated carbohydrates, the high iron diamine (HID) procedure (Spicer *et al.*, 1978) was performed on small blocks (2 mm on a side) of fixed tissue. The tissue was stained in a freshly prepared solution of high iron diamine for 24 h at 20°C and then rinsed twice in 0.075 *M* NaOH-PIPES buffer pH 6.8, and post stained for 1 h with 2% OsO_4 in the same buffer. These tissues were dehydrated, embedded and sectioned as indicated above.

Extracellular matrix materials were observed by the addition of 2% tannic acid to a fixing solution for fresh tissue consisting of 2% paraformaldehyde and 1.5% glutaraldehyde buffered with 0.075 *M* NaOH-PIPES pH 5.2. Post fixation was done in 1% OsO_4 for 1 h (Singley and Solursh, 1980). Dehydration embedding and sectioning was carried out as with other specimens.

Light microscopy

Fixed, isolated foot anterior cross sections were dehydrated in an ethyl alcohol series and then infiltrated with xylene. Xylene was substituted with paraffin and the tissues were cut into 10 μm thick sections and mounted on glass slides for cytochemical studies.

Alcian blue stains for determination of acid mucopolysaccharides were done by the methods of Pearse (1968). For detection of acidic mucosubstances, sections were exposed to freshly prepared 1% alcian blue 8 GX in 3% acetic acid for 30 min at pH 2.5. These sections were washed in running water, dehydrated in alcohol, cleared in xylene and mounted with Permount (Fisher Chem. Co., Fair Lawn, N. J.). Weakly acidic sulfated mucoids were stained with 1% alcian blue 8 GX in

0.1 *N* HCl for 30 min at pH 1.0. Sections were blotted with Whatman No. 50 filter paper and dehydrated, cleared, and mounted as above.

All light micrographs were taken on a Leitz Ortholux II interference contrast microscope equipped with a 35 mm camera.

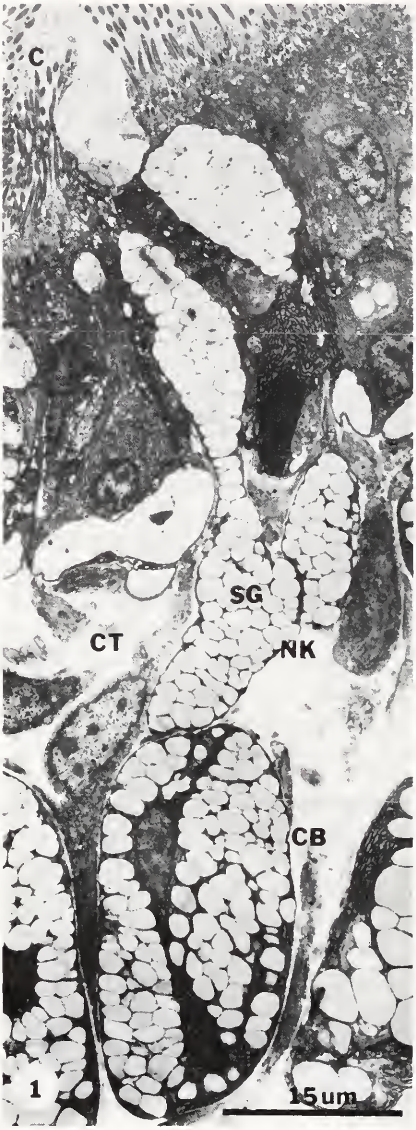
RESULTS

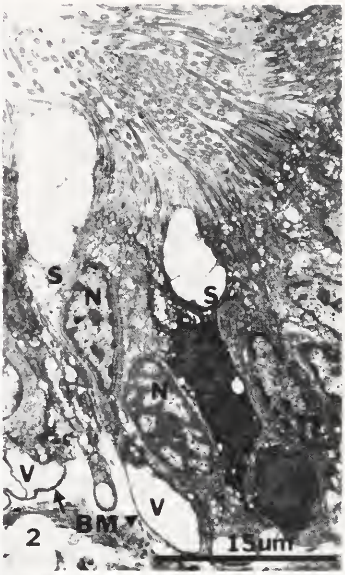
Ultrastructural morphology

The anterior sole of the foot in *Onchidoris muricata* consists of a ciliated secretory epithelium (Fig. 1). This epithelium is 20–30 μm thick and is a mixture of columnar ciliated cells and secretory gland cells. The narrow ciliated cells have a prominent centrally located nucleus and many mitochondria distributed in the cytoplasm toward the ciliated apex of the cell. A few vesicles are often present among the basal bodies of the cilia. In some of the ciliated cells large vacuolar spaces are present at the basal end near the basement membrane (Fig. 2). The secretory cells are usually wider than the ciliated cells and contain a greater variety of cytoplasmic organelles. Rough endoplasmic reticulum (ER), small vesicles and secretory granules are very common in the cytoplasm and easily detected. Golgi bodies are also present, but they are often out of the planes of section and, therefore, not visible in every cell. The basal vacuolar space found in ciliated cells is also present in the secretory cells (Fig. 1). In osmified tissues the product of the epidermal secretory granules is not easily distinguished from the product of subepidermal secretory cells. The neck or process of the subepidermal cells passing through the epidermis has contents similar to the mass of secretory granules of epidermal secretory cells (Figs. 1, 2). The two products are distinguished from one another with specific cytochemical stains (Figs. 8, 9). The epidermal cells rest on a basement membrane below which are various connective tissue cells surrounded by a considerable extracellular fibrillar component (Fig. 1).

The subepidermal long-necked secretory cells are found among the connective tissues below the basement membrane. All these cells are massed centrally in the foot and extend from one-third to two-thirds the distance from the anterior of the foot. The mass of long-necked cells is more widely distributed towards the anterior of the foot. The cell distribution then trails to a narrow point towards the foot posterior. The secretory cell bodies among the connective tissues are ovoids averaging 20 μm by 40 μm with the long axis perpendicular to the foot surface. A single narrow neck or process extends 45–70 μm from the cell body towards the surface of the foot. The neck penetrates the basement membrane and passes between the epithelial cells to the exterior of the foot (Fig. 1). The entire long-necked process contains tightly packed secretory granules that fuse as the secretory product is expelled from the apical end of the neck (Fig. 3).

The cell body also contains large numbers of secretory granules surrounding a centrally located nucleus, substantial amounts of rough ER, and a prominent Golgi body (Fig. 1, 4, 5). The secretory granule contents appear either fibrillar or granular (Fig. 5) in osmium post-fixed tissue. The Golgi body is usually found among the granules, commonly with progressively distending cisternae. The larger cisternae contained the fibrillar product which appear to change slightly into a granular material as the secretory granules mature and move distally from the Golgi body (Fig. 5). The proximal position of the expanding Golgi cisternae to the smaller secretory granules and the contents common to both organelles suggests secretory granule generation by the Golgi body (Fig. 5).





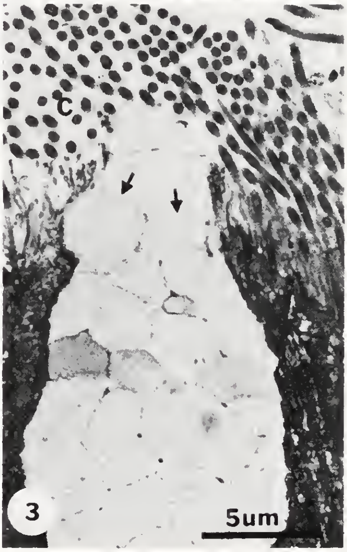
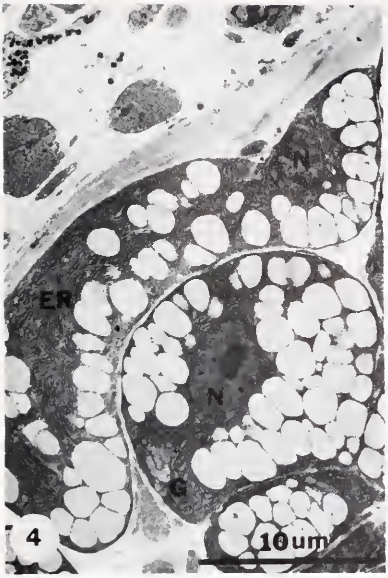
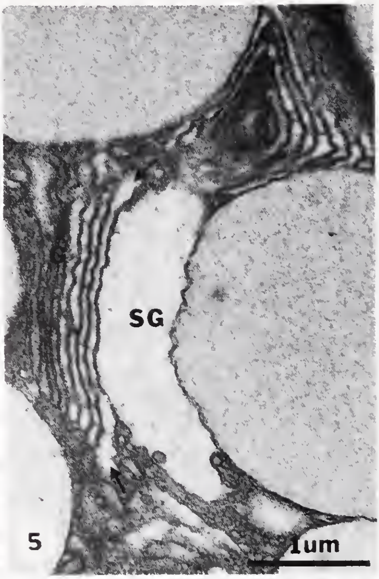


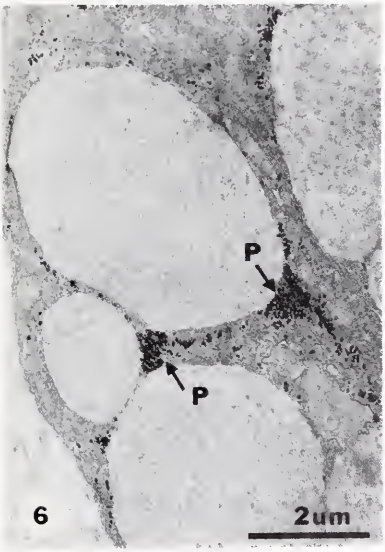
FIGURE 1. The foot epithelium of *Onchidoris muricata* showing the ciliated (C) surface cells and the long-necked secretory cells. These secretory cells have the cell body (CB) in the subepithelial connective tissue (CT). The process (NK) containing secretory granules (SG) extends to the surface of the foot.

FIGURE 2. The cells forming the epithelium have a basal vacuole (V). The nucleus (N) may be centrally or basally located in the cytoplasm. Some of these cells are secretory (S). The entire epithelium is subtended by a basement membrane (BM).

FIGURE 3. Expulsion of mucoid at the foot surface results from fusion of apical granules with plasmalemma and granules below, the separating membranes becoming obliterated (arrow). The mucoid aggregates and is dispersed by cilia (C).







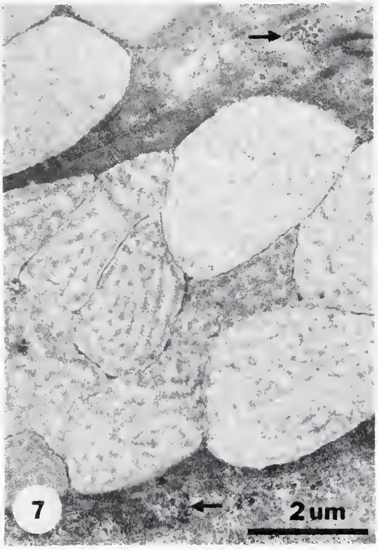


FIGURE 4. A cross section of the long-necked cell body showing the nuclei (N), large amounts of endoplasmic reticulum (ER), Golgi body (G), and secretory granules.

FIGURE 5. The Golgi apparatus (G) of the long-necked cell shows the distending cisternae (arrows) with contents similar to immature secretory granules (SG).

FIGURE 6. The periodic acid-thiosemicarbazide-silver protein (PA-TSC-SP) test is positive for glycogen (P, arrows), but not for the contents of the secretory granules. This test is negative for carbohydrates with obstructing groups.

FIGURE 7. TSC-SP control for the PA-TSC-SP test. Glycogen (arrows) is not positive when periodic acid oxidation is omitted from the test procedure.

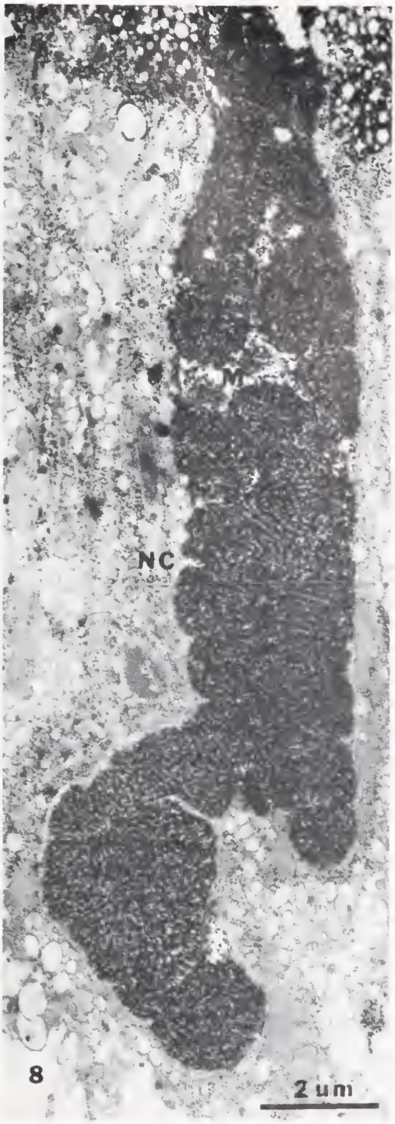
Cytochemistry

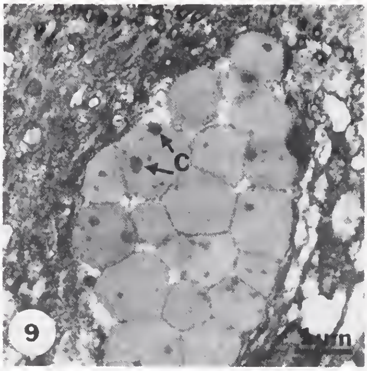
The contents of the long-necked secretory cells were analyzed for periodic acid oxidizable carbohydrate. The ultrathin sections were oxidized with periodic acid and then submitted to thiosemicarbazide for conversion to a derivative that precipitated silver metal from silver proteinate. Observations of cytoplasmic components within the long-necked cells showed that granules of α-glycogen were readily oxidized by this procedure and caused significant silver deposition (Fig. 6). The glycogen was often found concentrated around and in between the secretory granules, although smaller amounts were distributed throughout the cytoplasm (Fig. 6). The secretory material in the Golgi apparatus and granules did not show significant reaction in this test (Fig. 6). The fibrillar or granular structure in these organelles was similar to that found in nonoxidized osmium stained tissue (Fig. 5). Controls in which periodic acid oxidation was omitted, but thiosemicarbazide and silver protein included, showed no reaction in the glycogen deposits (Fig. 7). These results demonstrated that this test was specific for periodic acid oxidizable carbohydrate, as demonstrated by the dark staining glycogen. In addition, the PA-TSC-SP tests also showed that the secretory material within the granules did not contain significant amounts of this type of carbohydrate. Presumably, any carbohydrate present had obstructive groups preventing periodic acid oxidation.

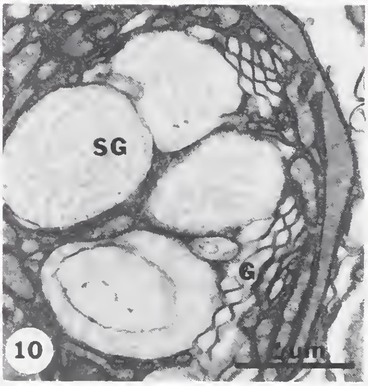
To test for possible sulfated polysaccharides that are not sensitive to periodate oxidation, the high iron diamine (HID) test was used on fresh foot tissue. This test is specific for complex carbohydrates esterified with sulfate. The regions occupied by secretory granules in the long-necked cells in osmified tissues were completely stained with HID (Fig. 8). In addition, the mucous blanket over the ciliated epithelium stained similarly (Fig. 8). This test also proved useful in distinguishing the long-necked cells from the shorter epithelial cells. The secretory products in the short cells (Fig. 9) did not stain with HID to the same level of intensity as those in the long-necked cells. Those granules in the short cells which had the greatest stain density appeared fibrillar with darker small cores among the fibrils (Fig. 9). Sometimes several of these cores were present in one granule. Generally all the secretory granules within a specific cell reacted similarly to this test. Thus, each cell type appeared to contain granules all having similar contents rather than mixtures of granules with different products (Figs. 8, 9).

The possible production of glucosaminoglycans by long cells for extracellular use was tested using tannic acid as a preservative for these compounds. The Golgi apparatus was free of any precipitated material, suggesting that these types of compounds were not present in this organelle (Fig. 10). Similarly, the granules arising from the Golgi body also were free of structures suggesting these compounds. Some secretory granules at the apical end of the cell near the plasmalemma were denser than others, but the density appeared to be related to the condensation of the fibrillar contents rather than to specificity of the fixative (Fig. 11). By comparison, the intercellular matrix appeared densely stained, as might result from the presence of intercellular matrix compounds such as hyaluronic acid or other glucosaminoglycans.

The use of alcian blue for staining sulfomucins at the light microscopic level complimented the results obtained with the electron microscope using the PA-TSC-SP and HID reactions. Alcian blue used at pH 2.5 and 1.0 stained the granular product of the long neck cells (Figs. 12, 13). Some of the epidermal cell granules were also stained, but not to the same density or extent as the long-necked cell granules (Fig. 12).







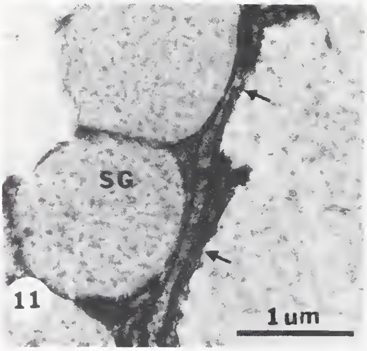


FIGURE 8. The long-necked cells (NC) and mucus (M) showing the intensity of high iron diamine (HID) staining. This stain is for sulfate groups on polysaccharides.

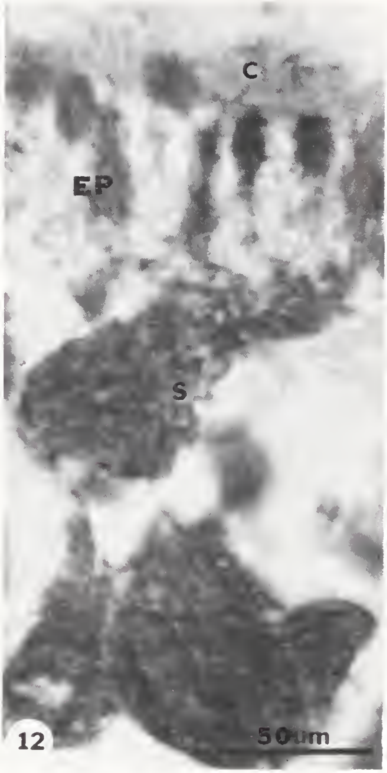
FIGURE 9. Secretory granules in the short epithelial cells show differences in HID staining. Dense cores in the granules (C) are more densely stained than the rest of the matrix.

FIGURE 10. Golgi bodies (G) and secretory granules (SG) do not stain when tissues are fixed with tannic acid. This procedure stains extracellular complex carbohydrates.

FIGURE 11. Tannic acid fixation and staining of intercellular carbohydrates (arrows).

DISCUSSION

The ultrastructure of mucous cells in marine invertebrates (Storch and Welsch, 1972; Cranfield, 1973a) and secretion in gastropods have been relatively little studied. Biochemically, a wide variety of mucopolysaccharides have been characterized



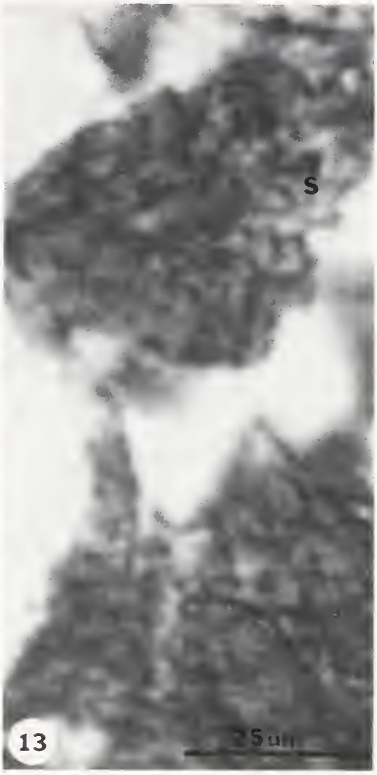


FIGURE 12. Alcian blue 8 GX stained granular product (S) in the long-necked cells. Cilia (c) of the epidermal cells cover the surface of the foot. Epidermal secretory cell (EP) products stain to a lesser extent. Contrast to stain enhanced with contrast interference optics.

FIGURE 13. Detail of several cell bodies of long-necked cells full of secretory product (S). Alcian blue 8 GX, pH 2.5 and 1.0 stain secretory products similarly.

from snails and slugs (Goudsmit, 1972). In most cases, the origin of these materials has not been narrowly defined, and the cells responsible for their secretion have not been identified. The paucity of such studies may have been caused by the confusing variety of secretory cell types distributed over the gastropod body and foot (Porter and Rivera, 1980).

Electron microscopic techniques greatly facilitate studies of individual cell types because the increase in resolution permits close examination of the cellular structures as well as the products of cytochemical reactions which the organelles undergo. The subepidermal long-neck cells in the foot of *Onchidoris* provided a good system for study of secretory cells because they appear isolated as a group and do not have a large variety of different cell types around them. These long-necked secretory cells are found in many nudibranchs (Hyman, 1967). The ultrastructure of these glands has been briefly described in the Mediterranean nudibranch, *Thuridilla hopei* (Storch and Welsch, 1972). However, secretory cells with long processes for directing and expelling secretions are not restricted to gastropods. The acetabular digestive gland cells in the cercarial life stage of schistosomes have a similar structure (Dorsey and Stirewalt, 1971).

The secretory product in the granules of the long-necked cells arises from the Golgi body. While there are considerable cisternae of rough ER in the cell body, no noticeable number of vesicles or granules appear to be closely associated with this organelle. The ER, Golgi body, and secretory granular components resemble those found in secretory cells of other gastropod tissues. Secretory cells in snail multifide gland (Ovtracht, 1967; Ovtracht *et al.*, 1969; Chailley, 1979) and snail hepatopancreas (Rivera, unpublished) have similar profiles. The relatively substantial amounts of rough ER probably contribute considerably to secretory product synthesis (Neutra and Leblond, 1966). In this respect, the long-necked cells resemble goblet cells of mammalian systems (Neutra and Leblond, 1966; Whaley, 1975). Oyster pediveligers also have similar gland cells (Cranfield, 1973a). In all these cases, the Golgi body appears to produce a single type of secretory product or mixture of products which results in similar granule structure throughout the cell. This nudibranch subepidermal foot cell differs from some types of body secretory epidermal cells in which the Golgi apparatus produces two different products (Porter and Rivera, 1980).

The cytochemical tests used to identify the contents in the secretory granules were selected to detect neutral, sulfated, and aminated polysaccharides. Cranfield (1973b) studied the settlement of oyster larvae and found an increase in the acidity of the secretions from the gland cells in the foot of the pedioveliger as it became sedentary. One type of gland cell (DI) in the oyster pediveliger has secretory granules that ultrastructurally resemble the long-neck cell granules (Cranfield, 1973a). The DI cell of the oyster may contribute to adhesion of the larva to the substratum (Cranfield, 1973b). Since *Onchidoris* travels with its foot on the substratum, some adhesion may be required. The presence of strong adhesives like glucosamnoglycans in the secretory granules was ruled out with the tannic acid tests. The use of tannic acid in the fixation procedure has been used successfully for detection of intercellular matrices during animal development (Singley and Solursh, 1980). Tissues of *Onchidoris* treated with tannic acid showed a strong positive reaction in the intercellular spaces. However, the absence of tannic acid reaction products in the Golgi apparatus and the secretory granules exclude the long-necked cells as producers of glucosaminoglycans.

Studies on epidermal mucoid secretory products in other nudibranchs show that the granules are PAS positive at the light microscopic level (Porter and Rivera, 1980). The granules in *Aeolidia papillosa* are particularly susceptible to periodic acid oxidation (Porter and Rivera, 1979). However, the secretory granules in *Onchidoris* do not contain periodic acid oxidizable neutral carbohydrates. This is substantiated by comparing the intensely stained glycogen in the cytoplasm of long-neck cells to the granules that remain unstained after the PA-TSC-SP procedure.

The high iron diamine (HID) test for detection of sulfated carbohydrates was used to indicate sulfate esters commonly found in molluscs (Goudsmit, 1972) and often known components in some types of mucous cells (Spicer *et al.*, 1978). This test has variable staining capacities of individual granules or of different granules within a granule population in a single cell (Spicer *et al.*, 1978). This variability in staining was observed in some of the epidermal secretory cells in the foot of *Onchidoris*. Thus, some variable amounts of sulfation are present in these secretory products. In contrast, the long-necked cells stained homogeneously and intensely. The light microscopic stains with alcian blue substantiate the ultastructural cytochemistry. Since alcian blue stains these secretory granules at both pH 2.5 and 1.0, the products may be interpreted as weakly acidic sulfated mucopolysaccharides (Pearse, 1968).

The sulfated polysaccharide in the long-necked cells of *Onchidoris* may provide lubricity for locomotion and possibly impart some degree of adhesion for attachment to the substrata. The ultrastructural cytochemistry of these cells is similar to skin epidermal secretory cells of the nudibranch *Coryphella rufibranchialis* (Porter, 1980). Adhesion is not a necessary property in this position on the body of *Coryphella.* However, lubricity for reduction of resistance is important. The similarity of the cellular product in these two organisms supports the lubricating function of the product. However, adhesive properties of this product cannot be excluded. The work of Cranfield (1973a; 1973b) on the settlement of oyster larvae indicates a cell type (D-1) with similar structure and chemical properties to the *Onchidoris* subepidermal foot cells. The oyster cell is functional during the active moving stage as well as the cementing stage of the larval life cycle (Cranfield, 1973b). If the product in *Onchidoris* has adhesive properties, they do not appear to be very strong. The ease with which *Onchidoris* may be removed from the substrata and the speed at which they move indicates a greater need of lubricant than adhesive.

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MECHANISM OF SPERM-OOCYTE INTERACTION DURING FERTILIZATION IN THE SURF CLAM *SPISULA SOLIDISSIMA*

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ABSTRACT

Spisula oocytes were treated with Triton X-100 to investigate sperm receptor(s) on the surface membrane. The study shows that macromolecules with sperm receptor activity reside on the surface membrane of oocytes. The evidence is that: 1.) Triton X-100 treated oocytes fail to undergo GVBD on exposure to sperm; 2.) Triton X-100 treated oocytes incubated with oocyte extract are capable of sperm-induced reactivation; 3.) Incubation of sperm with oocyte extract interfered with the fertilizing capability of sperm; 4.) Incubation of oocyte extract with sperm eliminated its ability to reactivate Triton X-100 treated oocytes to undergo GVBD; 5.) Reactivation of Triton X-100 treated oocytes is species-specific, *i.e., Spisula* oocyte extract was active, while *Arbacia* oocyte extract was inactive; 6.) the lectin that interacts with fucose blocked GVBD induced by sperm.

INTRODUCTION

Fertilization is a complex sequence of events that involves species-specific fusion of plasma membranes of the sperm and the egg. It is likely that the initial interactions between the sperm and the egg are mediated by surface macromolecules that facilitate species specificity and binding (Schmell, *et al.*, 1977). Although the morphological events involved in the sperm-egg fusion have been extensively studied (Franklin, 1965; Longo and Anderson, 1970; Giudice, 1973), little is known about the molecular aspects of this process. Studies in the sea urchin (Summers and Hylander, 1975) and mammals (Hartman and Hutchinson, 1974) have shown that sperm adhesion to the egg investing layer is a species-specific phenomenon. For many years it was assumed that the sea urchin jelly coat functioned as the sperm receptor during fertilization (Lillie, 1914). However, it has been shown by others (Tyler, 1948; Summers and Hylander, 1975) that interaction between the egg jelly coat and the sperm are not necessarily species-specific.

The purpose of the work reported here was to investigate whether there are sperm recognition macromolecules on the surface of the surf clam *Spisula solidissima* oocytes that are responsible for species specificity and binding during the fertilization process. The surf clam oocyte has a great advantage for study because it contains a large germinal vesicle with a membrane that breaks down readily on

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Abbreviations: Con A, *Concanavalia ensiformes* agglutinin; DBA, *Dolichos bifluorus* agglutinin; GVBD, germinal vesicle breakdown; PNA, peanut (*Arachius hypogaea*) agglutinin; RCA-1, *Ricinus comunis* agglutinin; SBA, soy bean (*Glycine max*) agglutinin; UEA, *Ulex europaius* agglutinin; WGA, wheat germ (*Triticum vulgaris*) agglutinin.

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fertilization (Schechter, 1941; Allen, 1953). Thus the breakdown of the nuclear membrane can be used to determine the occurrence of fertilization in oocytes.

To establish the presence of macromolecules responsible for recognition and binding, the following criteria need to be fulfilled: the sperm receptor macromolecules must be located on the surface of the oocyte, *i.e.*, either on the plasma membrane or on the vitelline layer, and be saturable; in addition, the interaction should be linked to biological activity (*i.e.*, GVBD) and species-specific; when isolated, the oocyte surface macromolecules should inhibit fertilization by binding to the sperm counterpart; oocytes denuded of the active macromolecules must fail to show fertilizability; addition of the removed oocyte surface macromolecules back to the denuded oocytes should restore fertilizability of these oocytes.

MATERIALS AND METHODS

Collection of gametes

Spisula solidissima and *Arbacia punctulata* were obtained from the Animal Resources Department of the Marine Biological Laboratory, Woods Hole, Massachusetts. They were maintained at 14°C in tanks containing running natural sea water. Sex was determined and large numbers of gametes were obtained by the method outlined by Allen (1953). Briefly, the two valves of the shell were separated by cutting the adductor muscles and removing the visceral mass intact. The gonads were exposed and excised.

Oocytes: The excised ovary was dissected in 200 ml of filtered sea water and strained through cheesecloth into a large beaker containing 200 ml of fresh filtered sea water. The oocytes were allowed to sediment to the bottom of the beaker and the supernatant removed. This process was repeated three times. The oocytes could be kept at room temperature for 2 h without deterioration.

Spermatozoa: The excised testes were also minced in 200 ml of filtered sea water and filtered twice through cheesecloth prior to storage on ice. Alternatively, "dry" spermatozoa were obtained by the method outlined by Allen (1953). Testes were incised and kept on ice for 1 h. At the end of this period, a milky sperm fluid had oozed out of the testis. This fluid was collected with a syringe and maintained on ice until used.

Fertilization of Spisula oocytes in vitro: *standardization of the Germinal Vesicle Breakdown Assay (GVBD)*. Germinal vesicle breakdown was determined by the methods of Schechter (1941) and Allen (1953). Using various concentrations of the sperm suspension against a fixed number of oocytes in Petri dishes containing filtered sea water, a sperm/oocyte ratio was reached at which maximum fertilization as determined by GVBD occurred after 10–15 min depending on the condition of the oocytes (Fig. 1). Percent GVBD was determined by counting the number of oocytes in a total count of 200 oocytes. The dishes were set up in duplicate. The variation between duplicate dishes was ±4%. To each dish containing approximately 5,000 oocytes in filtered sea water predetermined concentrations of spermatozoa were added. GVBD was determined by phase contrast microscopy.

Treatment with Triton X-100: An experiment to determine the effect of Triton X-100 on the fertilizability of *Spisula solidissima* oocytes was performed. Fixed amounts of oocytes were treated with various concentrations of the detergent and percent GVBD determined (Fig. 2). For most of the experiments, about 5,000 oocytes in 1 ml of filtered sea water were used. The oocytes were treated with detergent, washed three times by serial transfer into fresh filtered sea water dishes, and the spermatozoa added to the last dish containing the oocytes in duplicate.

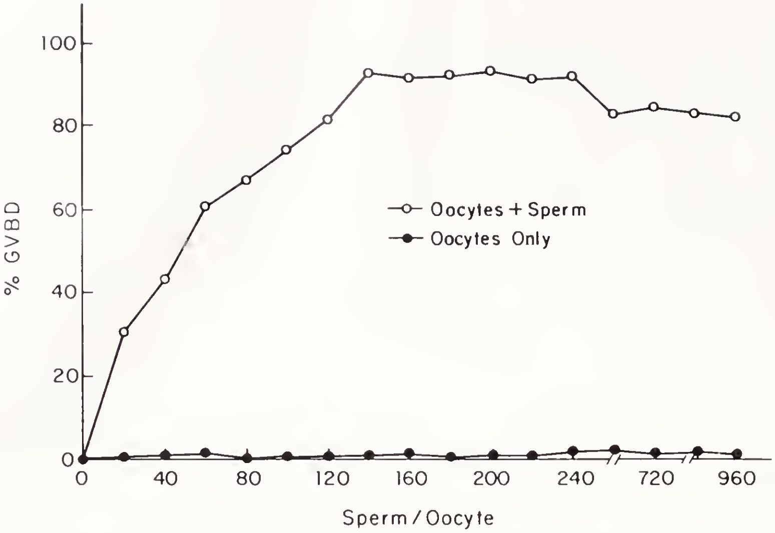

FIGURE 1. Induction of germinal vesicle breakdown (GVBD) at various concentrations of sperm per oocyte. GVBD observed 30 min after insemination at 25°C. (n = 4).

Preparation of oocyte extract: To investigate further what had happened to the *Spisula* oocytes after treatment with Triton X-100, a supernatant obtained from the detergent-treated oocytes was used to restore fertilizability to these oocytes. For this purpose the supernatant designated "oocyte extract" was treated as outlined in Figure 3. The supernatant was treated with several batches of SM-2 beads

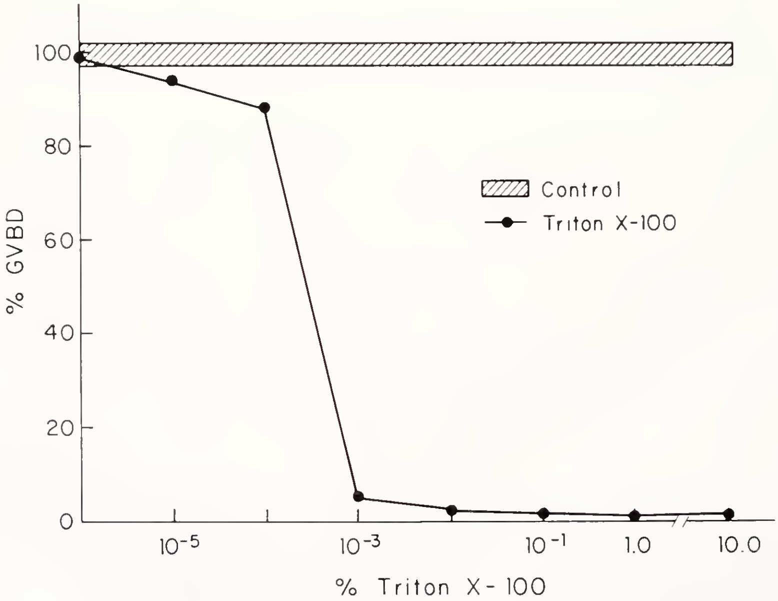

FIGURE 2. Effect of varying concentrations of Triton X-100 on GVBD in *Spisula* oocytes *in vitro*.

(Bio-Rad, Richmond, California) by the method of Holloway (1973) to remove Triton X-100. After concentrating with polyethylene glycol (PEG 20,000, Fisher Instruments) the total protein in the extract was estimated (Lowry *et al.*, 1951) and subsequently used in reconstitution experiments.

Viability of oocytes after treatment with Triton X-100: To determine whether the detergent at the concentrations used was cytotoxic to the oocytes, viability of the oocytes was established by observing the number of cells stained on exposure to 0.8% Trypan blue after serial transfer washing (Fig. 4).

Reconstitution experiments

(a) *GVBD in detergent-treated oocytes after the addition of the processed "receptor extract."* To determine what had occurred to the oocyte surface, the detergent-treated and washed oocytes designated as "stripped" oocytes (Fig. 3) were incubated with the oocyte extract containing different amounts of protein, for 60 min, washed by serial transfer and the GVBD determined.

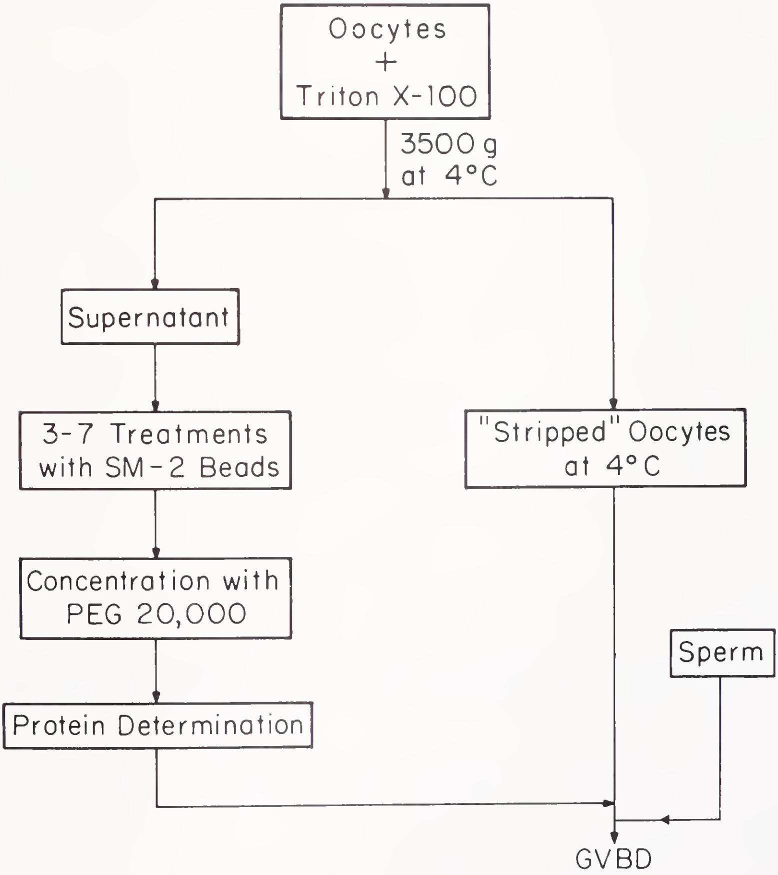
Oocytes
+
Triton X-100
3500 g
at 4°C
Supernatant
3-7 Treatments with SM-2 Beads
Concentration with PEG 20,000
Protein Determination
"Stripped" Oocytes at 4°C
Sperm
GVBD

FIGURE 3. Outline of the procedures used for the extraction and processing of *Spisula* oocyte "receptor extract" used in the reconstitution experiments. PEG—polyethylene glycol.

(b) *Species-specificity of oocyte receptor extract in the restoration of GVBD.* To test the hypothesis that a molecular species removed from the oocyte surface by detergent was involved in species-specific recognition and binding, a soluble receptor extract was prepared from the sea urchin *Arbacia punctulata* oocytes using a modification of the technique of Schmell and colleagues (1977). *A. punctulata* oocytes were obtained by the electrical method of Harvey (1956). The oocytes were incubated at room temperature for 1 h and washed three times with freshly filtered sea water. They were lysed by suspension in distilled water by centrifugation for 10 min at 1000 × *g*. This process was repeated. The resulting pellet was resuspended in freshly filtered sea water and homogenized by 10 gentle strokes in a ground glass homogenizer. The resulting homogenate was dialysed overnight against freshly filtered sea water at 4°C and a soluble oocyte receptor extract obtained by centrifugation of the retentate for 30 min at 12,000 × *g*. Both the supernatant and the pellet were stored at −60°C until used. To determine whether the supernatant or the pellet contained "receptor activity" both were tested for ability to restore fertilizability to trypsin-treated *Arbacia punctulata* oocytes (Schmell *et al.*, 1977). Both the supernatant and the pellet were found to contain a component that could restore fertilizability to the reconstituted *Arbacia* oocytes. Only the supernatant was used in reconstitution experiments with *Spisula.*

(c) *Effect of the* Spisula *oocyte receptor extract on the capability of* Spisula *sperm to induce GVBD in normal oocytes.* The reduction of GVBD in oocytes after treatment with detergent not only suggests the existence of surface molecules on the oocyte, but also the presence of complementary macromolecules on the sperm surface. If *Spisula* spermatozoa contain specific surface molecules that combine with complementary molecules on the oocyte surface in a key-and-lock fashion, then the treatment of normal spermatozoa with the oocyte receptor extract should block GVBD. Before testing this hypothesis, the capability of the oocyte receptor extract to bind to normal sperm was determined. Spermatozoa (5×10^3) in 1 ml of filtered sea water were incubated for 30 min with the oocyte extract, centrifuged twice for 10 min at 12,000 × *g*, and the supernatants used for the restoration of GVBD in detergent-treated oocytes.

(d) *Treatment of oocytes with lectins*: To determine what type of sugars are involved in *Spisula* oocyte-sperm interaction, oocytes were incubated with 100 μg/ml of various lectins for 30 min, washed and percent GVBD determined following insemination.

RESULTS

Germinal vesicle breakdown assay: An asymptotic curve was obtained when experiments were done to determine optimal ratio of sperm/oocyte concentrations at which maximum GVBD would occur. One representative experiment is depicted in Figure 1. The ratio of sperm to oocyte increased until there were about 140 spermatozoa per oocyte. Higher sperm/oocyte ratios did not significantly improve percent GVBD.

Effect of Triton X-100 on germinal vesicle breakdown: Figure 2 shows that Triton X-100 concentrations greater than 0.0001% inhibited GVBD in *Spisula* oocytes.

Viability: Viability of the detergent-treated oocytes was unaffected by concentrations of Triton X-100 lower than 0.0001% (Fig. 4).

Reconstitution experiments: Reconstitution experiments indicated that incubation of "stripped oocytes" with the oocyte extract restores GVBD to some oocytes

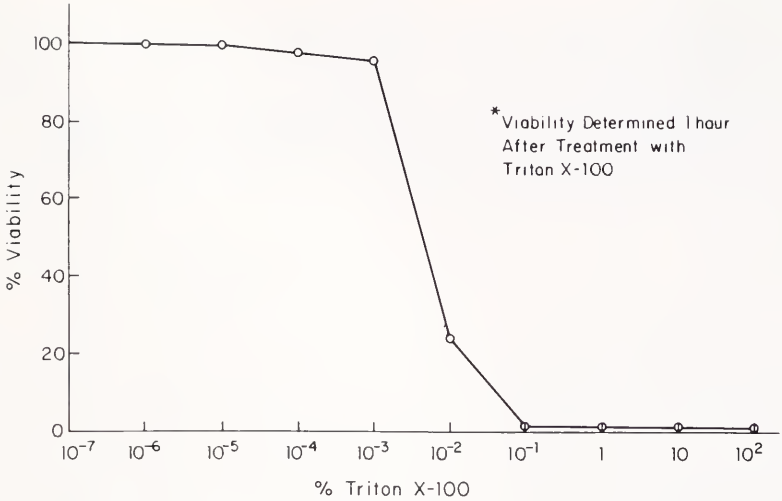

FIGURE 4. Viability of *Spisula* oocytes treated with varying concentrations of Triton X-100. Viability determined 60 min after Triton X-100 treatment by observing uptake of trypan blue. T = 22–24°C.

after 60 min (Table I), and that the heat-treated extract failed to restore GVBD capability to Triton X-100 treated oocytes while untreated extract restored GVBD to 21.6% (Fig. 5). Incubation of the "stripped oocytes" with the oocyte extract for longer than 60 min did not significantly improve percent GVBD. The percentage of reconstituted oocytes that underwent GVBD ranged from 15.4 ± 2.6 to 23.7 ± 3.9 (6 experiments) (Mean ± S.D).

Species specificity: Table II shows that only the *Spisula* oocyte membrane extract restored GVBD capacity to "stripped" *Spisula* oocytes. An *Arbacia* extract failed to restore GVBD capacity to "stripped" *Spisula* oocytes, suggesting that the ability of *Spisula* oocyte receptor extract to restore GVBD capacity to "stripped" *Spisula* oocytes may be species-specific.

Complementary sperm receptor: Pre-treatment of the *Spisula* oocyte extract with *Spisula* spermatozoa to absorb the sperm interacting proteins abolished the ability of the extract to restore GVBD capacity to stripped oocytes but had no effect on sperm motility (Table III). Triton X-100 treatment might have induced acrosome reaction in the sperm and reduced the fertilizing capacity. In addition,

TABLE I

Influence of oocyte extract containing sperm receptor on germinal vesicle breakdown in Triton X-100-treated Spisula *oocytes induced with sperm. GVBD in untreated oocytes, 95–98%.*

	GVBD (%)	
Incubation period (min.)	With extract (0.4 mg protein/ml)	No extract
15	2	0
30	4.3	0
60	17.8	1
120	16.9	1

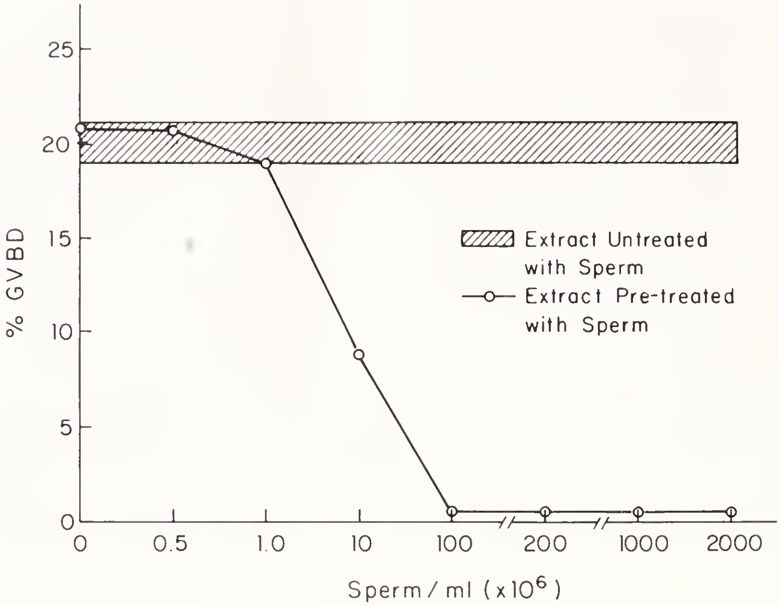

FIGURE 5. Capacity of oocyte extract absorbed with *Spisula* sperms to induce GVBD in "stripped oocytes." Varying concentrations of *Spisula* sperm were added to each ml of oocyte extract at 22°C for 30 min. The sperm were separated by centrifugation at 17,000 *g* for 10 min. The supernatant was tested for its capacity to restore the ability to undergo GVBD in Triton X-treated oocyte upon insemination. (n = 3).

the spermatozoa that had been pre-treated with the extract were unable to induce GVBD in normal oocytes (Table III and Fig. 5).

Inhibition of GVBD by lectins: Table IV shows that of the lectins tested for their involvement in sperm-oocyte interaction, only UEA with specificity for α-fucose was found to be directly involved. Treatment of the oocytes with 100 μg/ml of UEA, prior to insemination, reduced GVBD by 50%.

DISCUSSION

The rationale for conducting a study to determine the effect of Triton X-100 on *Spisula* oocytes was based on the premise that the oocyte surface contained

TABLE II

Influence of heterospecific oocyte extract containing sperm receptor on GVBD in Triton X-100-treated Spisula *oocytes.*

	GVBD (%)	
Incubation period (min.)	Sea urchin extract (0.32 mg protein/ml)	*Spisula* extract (0.4 mg protein/ml)
15	0	6.0
30	0	9.0
60	0	13.6
120	0	13.9

TABLE III

Influence of oocyte extract containing "sperm receptor" on sperm motility and fertilizability of Spisula *oocytes. Sperm preincubated with oocyte extract for 30 min and washed before use.*

	Sperm motility (%)		GVBD (%)	
Experiment no.	With extract (0.32 mg protein/ml)	No extract	With extract (0.42 mg protein/ml)	No extract
1	62	71	12	76
2	58	64	7	71
3	74	69	9	81

sperm-recognizing macromolecules and that these macromolecules could be extracted by the detergent without destroying their biological activity (Maddy and Dunn, 1976). Removal of macromolecules such as insulin receptor (Cuatrecasas, 1972, 1974), cholinergic receptor (Meunier, *et al.*, 1974) in biologically active form has been achieved. The present results show that treatment of oocytes with Triton X-100 removed a molecule or set of molecules necessary for recognition and binding. This is further supported by the sperm-oocyte ratios necessary for GVBD to occur as depicted in Figure 1. The asymptotic part of the curve implies saturation of the oocyte surface with sperm, *i.e.*, the oocyte surface has only a fixed number of sites to which spermatozoa can bind.

It is not clear whether the sperm binding sites or macromolecules on the *Spisula* oocyte are located primarily on the vitelline layer or on the oolemma which is projected into branched microvilli extending into the fibrous vitelline layer. A narrow perivitelline space extends from the oolemma to the lower edge of the vitelline layer. Figure 6 is a diagrammatic representation of an unfertilized *Spisula* oocyte drawn from an electron micrograph (Longo and Anderson, 1970). The electron micrograph shows that some of the microvilli extend to the surface of the vitelline layer. The exact localization of the receptor(s) was not determined although studies in the sea urchin (Aketa, 1975) suggest that they are located only on the vitelline layer.

Further evidence that specific macromolecules on the oocyte surface are involved in sperm-oocyte interaction was provided by our reconstitution experiments. About 20% of the oocytes were viable and able to undergo GVBD after re-incubation with

TABLE IV

Effect of lectins on GVBD in Spisula *oocytes. Oocytes pre-incubated with lectins (100 μg/ml) for 30 min and washed before use.*

Lectin competitor	Sugar specificity	GVBD (%)
Con A	α-D-Mannose, α-D-Glucose	98.0
PNA	D-Gal-β(1,3)-GalNAc	100.0
DBA	N-Acetyl-α-D-Galactosamine	95.0
RCA-I	β-D-Galactose	100.0
SBA	N-Acetyl-α-D-Galactosamine D-Galactose	99.0
WGA	N-Acetyl-β-(1,4)-D-Glucosamine 2(sialic acid)	100.0
UEA	α-L-Fucose	47.4

Controls ranged from 95.5–100%; 200 sperm/oocyte in each culture dish.
Values are the mean of 4 separate determinations.

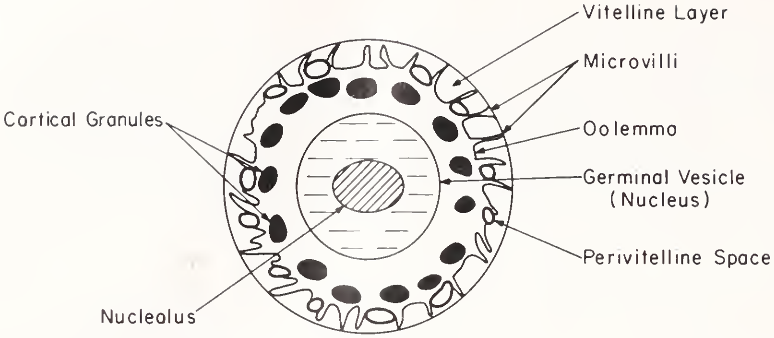

FIGURE 6. Diagrammatic illustration of an unfertilized *Spisula* oocyte. Drawing based on electron micrograph by Longo and Anderson (1970) showing possible sperm receptor sites on the oolemma or vitelline layer.

oocyte extract (Table I), suggesting that only a few oocytes can be reactivated to undergo GVBD. Perhaps the detached macromolecules underwent conformational changes and lost their capacity to reattach to their original or other corresponding sites, or the vitelline membrane was drastically altered. Membranes of a number of oocytes treated with 0.0001% Triton X appeared disrupted when viewed under the scanning electron microscope, indicating that these cells probably sustained irreparable injury.

One criterion for a molecule or set of molecules to act as a sperm receptor is species-specificity. Indeed, although human sperm will bind to hamster eggs *in vitro*, they will not fertilize these eggs (Gwatkin, 1976). It has been extensively shown that cross fertilization, though not absolute, fails to occur readily between different species of sea urchin (Kato and Sugiyama, 1978; Tyler and Tyler, 1966). The present finding that *Arbacia* egg receptor extract did not promote GVBD in detergent-treated *Spisula* oocytes supports the thesis that recognition and binding were species-specific processes.

Several workers have shown that in sea urchin (Aketa, 1973; Lallier, 1972) and in mammals (Gwatkin, 1976) Con A inhibits fertilization of eggs and that a receptor obtained from the egg surface binds to Con A (Schmell *et al.*, 1977). This finding suggests that the oocyte surface receptor contains either α-glucosyl or α-mannosyl units. When *Spisula* oocytes were treated with 100 μg/ml of various lectins, only UEA affected the capacity of the oocytes to undergo GVBD. The other lectins had no significant effect (Table IV). This indicates that α-fucose is involved in the sperm-oocyte interaction and that the fucose-containing molecule is located on the sperm. The involvement of fucose residues in sperm-egg interaction of *Ciona intestinalis* has been reported (Rosati *et al.*, 1978; Rosati and DeSantis, 1980).

The nature of the receptive molecule(s) on the egg suface is beginning to be explored. Early results in the sea urchin (Hagstrom, 1956) suggested that the receptor was altered by digestion with trypsin-like proteases. This was confirmed by Vacquier *et al.* (1972) and Aketa *et al.* (1968) who showed that digestion of the sea urchin egg surface with trypsin decreased fertilizability. A putative receptor protein was isolated from the surface of the sea urchin *Hemicentrotus* which when incorporated into air bubbles led to the binding of sperm to the bubbles (Aketa *et al.*, 1968). Although this material was not purified to homogeneity, it appeared to be a 2.3 S glycoprotein. Antiserum directed against this component blocked fertilization (Aketa and Onitake, 1969) and sperm treated with the component lost their fertilizing ability but not their motility (Aketa, 1973). Furthermore, sea urchin sperm treated with the component did not agglutinate or undergo the acrosome reaction. Thus the factor seems to bind sperm before the acrosome reaction occurs. This makes it less likely to be the component involved in species-specific binding although the antisera directed against it blocks fertilization in a species-specific fashion. This material seems to have some, but not all, of the binding characteristics expected from physiological, morphological and from *in vitro* and *in vivo* studies of sperm-egg interaction.

A similar approach was used to detect a component from hamster zona pellucida that was suggested to be involved in the binding reaction (Hartman *et al.*, 1972; Gwatkin and Williams, 1977). The component showed some cross-reactivity in the mouse system. More recently, a glycoprotein in mouse egg zonae pellucidae with receptor activity for sperm has been identified. The glycoprotein from zonae pellucidae of unfertilized eggs called ZP3 reduced the binding of sperm to eggs *in vitro* (Bleil and Wassarman, 1980).

Another factor suspected of being a sperm receptor of the egg has been obtained from a membrane protein preparation of *Arbacia* eggs that inhibited fertilization of *Arbacia* but not *S. purpuratus* (Schmell *et al.*, 1977). Trypsin treatment of the factor destroyed its blocking potential. The soluble form of the factor binds to sperm and to Con A. The *Spisula* oocyte membrane material, however, does not bind to Con A. These observations suggest that the oocyte surface factors of *Spisula* and *Arbacia* contain different carbohydrate moieties.

An approach similar to the one employed in our work to obtain a sperm receptor was reported (Vacquier and May, 1977; Glabe and Vacquier, 1978). An attempt was made to isolate the vitelline layer by Triton X-100 and EDTA treatment of eggs. These vitelline layer preparations contained microvillous projections similar to those seen on the egg and sperm adhered to them only on the external surface. When the vitelline layer preparations were iodinated and examined by polyacrylamide gel electrophoresis, two high molecular weight components were seen. A high molecular weight, trypsin-sensitive glycoprotein obtained from this preparation (Glabe and Vacquier, 1978) has specific affinity for binding a particular component of a Triton X-100 extract of sperm. This would agree with our finding in *Spisula* that a complementary receptor molecule to the oocyte receptor exists on the sperm surface.

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Continued on Cover Three

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DISEASE PROCESSES IN SEASTARS: A METCHNIKOVIAN CHALLENGE*

FREDERIK B. BANG

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ABSTRACT

The adult seastar is an ideal model for study of phagocytosis and inflammation. The behavior of the circulating amebocytes responsible for phagocytosis and clumping can be directly observed in the coelomic fluid circulating in the transparent papulae of the limbs. Intracoelomic injection of an autologous amebocyte extract induces temporary, reversible, clumping and adherence of amebocytes. Injected, foreign, red-pigmented sea urchin amebocytes are phagocytosed by seastar amebocytes which then form clumps in the tips of the papulae and traverse to the external surface through penetrating lesions. Successive injections may cause acute inflammation and edema. During the acute inflammatory response, a substance(s) is released into the coelomic fluid which stimulates the urn cell complex of *Sipunculus nudus* to secrete mucus *in vitro*; the substance disappears from the fluid by 24 hours. A spontaneous ciliate infection of seastar testes causes failure of the infected animal's amebocytes to clump on glass or plastic. Seastars recognize foreign grafts, but the role of amebocytes in the rejection has not been shown to be explicit. In the separate water vascular system of seastars, a secondary inflammatory response (amebocyte clumping) is induced by injections of bacterial suspensions and by repeated injection of sea urchin amebocytes into the coelom. Heavy exposure of seastars to ciliate populations induces appearance of a lysin which is not derived from circulating cells.

INTRODUCTION

Although Metchnikov** (1892) first tested his basic idea concerning phagocytosis and inflammation in seastar larvae, the mature seastar has been used only sporadically as an experimental animal for the study of inflammation. It is the purpose of this informal review to describe some recent experiments which show that inflammatory changes may be easily followed in the transparent papulae of the adult seastar. Unique changes in macrophage (amebocyte) behavior are also induced by spontaneous disease in the adult animal.

In the years following Metchnikoff's observations of spontaneous phagocytosis of diatoms and debris by *Bipinnaria larvae* (Metschnikoff, 1883) and the famous

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Abbreviation: MSS, mucus-stimulating substance.

* This article is based on a paper presented at the Symposium on Phagocytosis—Past and Future, at Messina (Italy), 22–27 September 1980, and the seastar section of a paper prepared for the Symposium on Immune Reactions to Parasites, at Mainz (Germany), 7–11 October 1981.

** For the spelling of the name Metchnikoff, each of the original references was used.

Editor's Note: Following solicitation of an article in the area of invertebrate pathobiology, Dr. Bang submitted a draft outline of this paper for the Editor's consideration. After Dr. Bang's untimely death on October 3, 1981, Mrs. Betsy Bang prepared the formal manuscript for publication.

experiment with the rosethorn (Metchnikoff, 1905), there were apparently very few further pathological studies on seastar larvae. Metalnikov and Rapkine (1925) reported in the *Comptes Rendus* that injection of India ink into 16–18 hour blastulas of sea urchins (*Paracentrotus lividus*) was followed by phagocytosis of the ink 3–4 hours later. Similar results were obtained with three species of bacteria, although *Vibrio cholerae* killed these larvae. Since that time, embryologists have frequently noted the ingestion of foreign material by larvae, but as far as I know there has been no systematic study of these processes. The transparent two-day old *Asterias* larva, which is capable of ingesting small clams (Mead, 1899) might be an ideal subject.

In contrast to what has been done on the structure and physiology of echinoderms (Cuénot, 1948; Hyman, 1955; Boolootian, 1966), there is only a scattering of information on pathological changes in the adult seastar. Within this context, we will describe the three circulatory systems of *Asterias* which have been used for experimental studies, and then describe different phenomena which directly affect the circulating amebocytes found in the coelomic cavity and in the water vascular system. In Metchnikovian terms, the transparency of intact animals is of great advantage for continuous observation of changes in individual experimental animals, and spontaneous infections offer visible insights into experiments of nature.

Results and Discussion

The seastar as experimental animal

The phenomena which we have examined in adult seastars are: (i) reversible clumping of the amebocytes in the papulae; (ii) normal clumping of the amebocytes when they are placed on glass, and inhibition of this clumping in amebocytes taken from seastars which have a natural ciliate infection in the testes (less frequently in the ovaries); (iii) recognition of foreignness of experimentally injected material followed by phagocytosis and emigration of phagocytosed material through the papulae; and (iv) rejection of foreign grafts, either of the hepatopancreas or of the skin. In all cases, the amebocyte is probably involved, but lytic factors have also been demonstrated under certain conditions.

Two important precautions are essential in working with adult seastars experimentally. First, animals must be healthy. Stars that have been dragged across the sea bottom on a mop during dredging lose a great deal of surface skin and usually have marine bacteria circulating within the coelom; many will subsequently autotomize one or more limbs (Bang and Lemma, 1962). Therefore, the animals must be collected by hand. In the Woods Hole area, *Asterias forbesi* can be handcollected by divers. In northern France, *Asterias rubens* can be collected directly off rocky mussel beds at low tide. The coelomic fluid of healthy animals is free of bacteria, and clumping is absent from the papulae.

Secondly, transparency varies with the species and age of an animal. *Asterias forbesi* which weigh 50 g or more do not have transparent feet, but in 6–10 g stars circulating cells are readily seen as they are driven by the internal cilia in the papulae and the feet. Present-day dissecting microscopes, with intense, relatively cold, lights, reveal to the practiced observer a wealth of circulating cells rushing through these respiratory and excretory papulae and feet.

I. Anatomy

For the present purpose, a review of only the simplest aspects is necessary. A cross-section of a limb shows the papulae emerging all over the surface, predom-

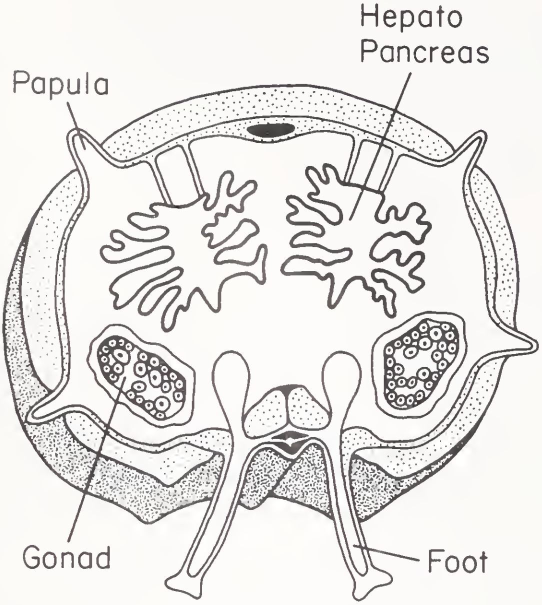

FIGURE 1. Cross-section of a seastar.

inantly on the dorsal or aboral surface (Fig. 1). These have a layer of epidermal cells and endodermal cells, both of which are ciliated, and a mesenchymal layer which contains contractile muscle cells. The papulae may be contracted or relaxed either in response to their own muscular action or to the general pressure of the body wall. We have often seen a general collapse of the usually distended papulae of a 10 g seastar immediately after a second injection of a foreign substance such as ciliate-infected crab blood, or sea urchin blood. In extreme cases, the change in pressure pulls the inverted papulae into the coelomic cavity. The papulae may also be grossly distended during edema. The coelomic fluid of normal *Asterias* contains only one type of cell, the petaloid cell, which circulates everywhere in response to both muscular movements of the whole arm and the constant directional beating of the cilia. The water vascular system is separate. Injection of particulates, such as India ink or carmine powder, into the coelom is not followed by their appearance in this second system. Even dyes, such as Evans' blue (Noble and Gregerson, 1946), penetrate from the coelom into the feet only after a number of days, and some of this may be by cell transfer. The third system, the axial organ and the hemal canals, is less well understood and reference to their role in pathology will be incidental.

II. Responses to injury

The use of natural responses to injury or infection among invertebrates was a hallmark of Metchnikoff's work on invertebrates. Not only was the yeast infection of *Daphnia* (Metschnikoff, 1884) studied extensively, but even during the original study of the metamorphosis and development of seastar larvae, he was careful to pay attention to the presence of foreign material that had been ingested by the larvae during their residence in the sea (Metschnikoff, 1883).

TABLE I

Prevalence of non-clumping amebocytes among Asterias *during summer 1976.*

Month/date	Number tested	Number negative for clumping	Per cent negative
6/21–23	38	2	5.3
6/29–30	40	3	7.5
7/ 6–12	50	18	36.0
7/22–8/4	126	6	5.0
8/11–14	179	3	1.7
8/17–19	81	8	10.0
8/19–23	197	0	0.0
9/ 3–6	110	1	0.9

a. Clumping. Some years ago, the fact that traumatized stars often showed clumps of cells circulating in the papulae and that in cases of severe trauma these clumps may be tightly contracted and become attached closely to the inside of the papulae, led us to make crude extracts of the amebocytes and to inject the fluid extract into the coelomic cavity (Bang and Lemma, 1962; Bang, 1975). Direct observation of the papulae showed an initial loose aggregation of the cells, later clumping and contraction of these aggregates, and the final tight adherence of balls of amebocytes to the inside of the papulae. This phenomenon occurred within 2–5 minutes, lasted only 15–20 minutes, and then disappeared, leaving the animals susceptible to repeated stimuli of the same kind without any apparent change in sensitivity.

b. Failure of amebocytes to respond to glass. A well described ciliate infection of the gonads (especially of the testes) of seastars was first observed in *Asterias rubens* by Cépède (1910), subsequently studied in *Asterias forbesi* in the U.S. by Smith (1936), and in England by Vevers (1951). It attains sufficient frequency under certain conditions that it was seriously considered by Galtsoff and Loosanoff (1939) as a method of controlling seastars which prey on shellfish beds. The apparently indirect effect of the infection on the glass-induced clumping of seastar amebocytes was discovered by one of my students in 1970, when he found that the routine clumping of cells on glass did not occur in animals in which the ciliate (*Orchitophyra stellarum*) was present in the gonads (Childs, 1970). Subsequently, I have found in the summers of 1976, 1978, and 1980, and even in January 1978, 1979, and 1980, that this failure of cells of infected individuals to react to glass has been readily demonstrable. In all four summers, it has appeared at high prevalence (about 30%) in animals from different sites near Woods Hole, Massachusetts (Table I). The very high correlation with ciliate infection (Table II) suggests that

TABLE II

Presence or absence of ciliates in seastars Asterias forbesi *and* A. vulgaris *combined.*

	Number examined	Number with ciliates	Percentage
Clumpers	57	0	0
Non-clumpers	24	22	92
Doubtful	6	1	17

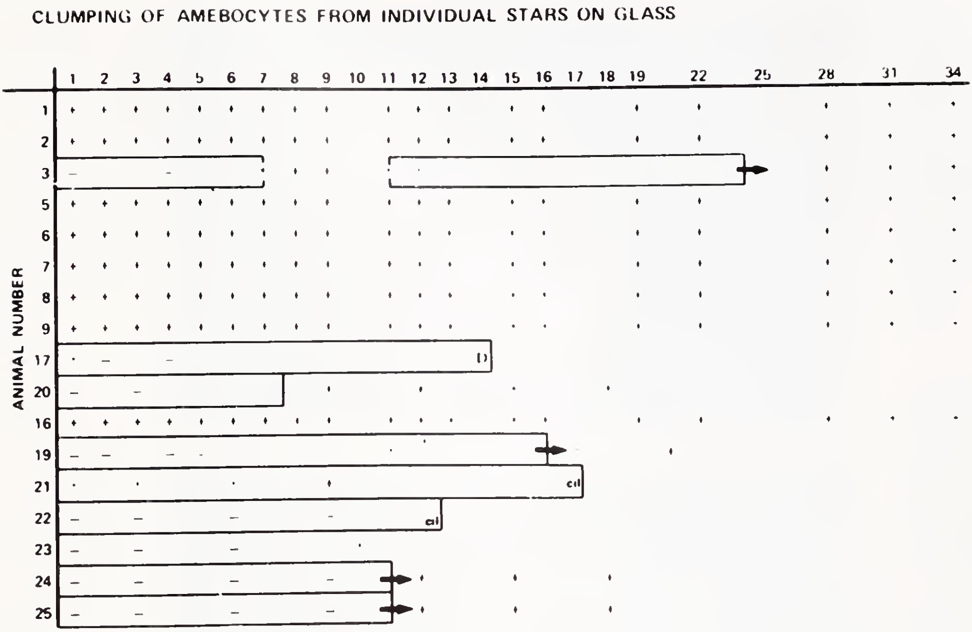

FIGURE 2. Clumping of amebocytes from individual stars on glass.

it is the direct cause of the abnormal behavior of these cells. Recovery from infection and return to normal glass-induced clumping frequently occurs within 10 days to 2 weeks (Taylor and Bang, 1978; Bang and Childs, unpublished) (Fig. 2).

The remarkable failure of the amebocytes to react to glass needs extensive study. While normal amebocytes begin to form clumps with each other within less than a minute after exposure to glass or plastic surfaces, cells from infected animals, whether obtained from the coelomic cavity or the water vascular system, remain unreactive (Fig. 3a,b,c). After centrifugation into a pellet, they can be readily resuspended by shaking and may be washed several times and even transferred into normal coelomic fluid without losing their abnormal surface properties. They do, however, gradually settle on the surface of a glass slide, and do ingest foreign material such as carmine or sea urchin cells injected into the coelomic fluid. However, the secondary reaction of sticking to each other is apparently inhibited completely. There seems to be a relation between heavy infections and the presence of the ciliates in the coelomic fluid. The similarity between amebocytes and mammalian platelets (Levin and Bang, 1964), noted at the turn of the century (Dekhuyzen, 1901; Loeb, 1902), is a still unexploited challenge for investigation of the surface properties of these cells.

III. Inflammation

A recent review by Ryan and Majno (1977) defined inflammation as a response of living tissue to local injury that leads to the local accumulation of blood cells and fluid. The overall process, seen against the broad perspective of evolution, is useful since its primary function is doubtless that of defense against microscopic invaders. Metchnikov (1892) in his "*Lectures on the Comparative Pathology of*

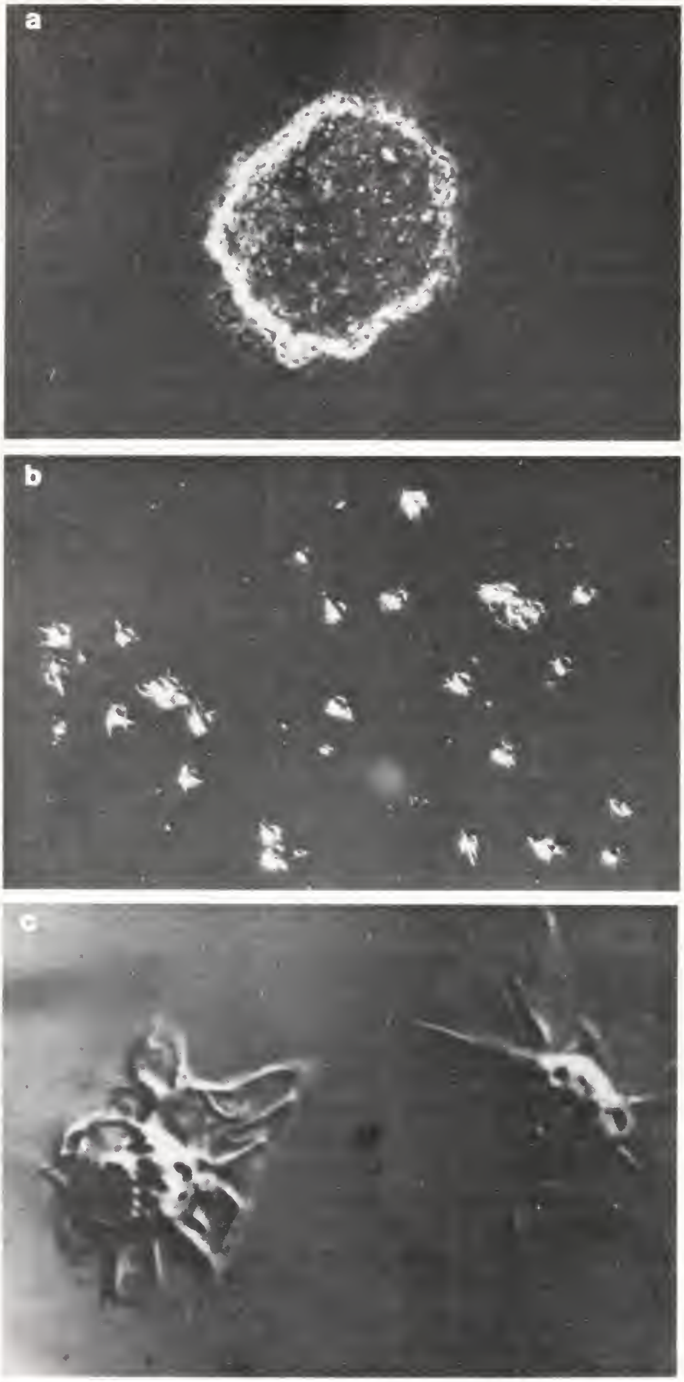

FIGURE 3. (a): Blood from a normal seastar forming a tight clump of amebocytes when exposed to glass. (b): Blood from a ciliate-infected seastar failing to clump on exposure to glass. (c): Individual amebocytes from ciliate-infected star showing normal petaloid form. (Nomarski optics) [Courtesy Dr. K. Edds.]

Inflammation" defined inflammation in terms of phagocytosis. At about this same time, when there was considerable interest in the excretion of foreign substances by invertebrates, Durham (1888) studied the emigration of ink-laden amebocytes from the seastar through the transparent papulae.

A. Response to sea urchin blood cells. We found that injection of sea urchin blood cells into the seastar was also followed by clumping of the urchin cells into cell plugs which appeared at the tips of the papulae 4–10 hours after injection (Reinisch and Bang, 1971). There was a concomitant marked reduction in the number of circulating host amebocytes, an effect that could be transferred to other

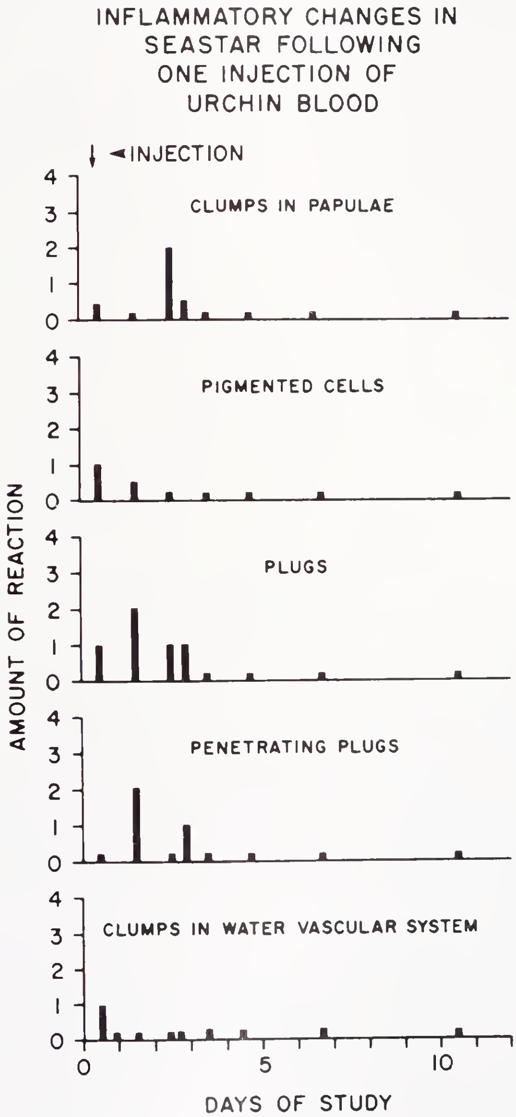

FIGURE 4. Inflammatory changes in seastar following one injection of urchin blood. Small bars represent negative findings.

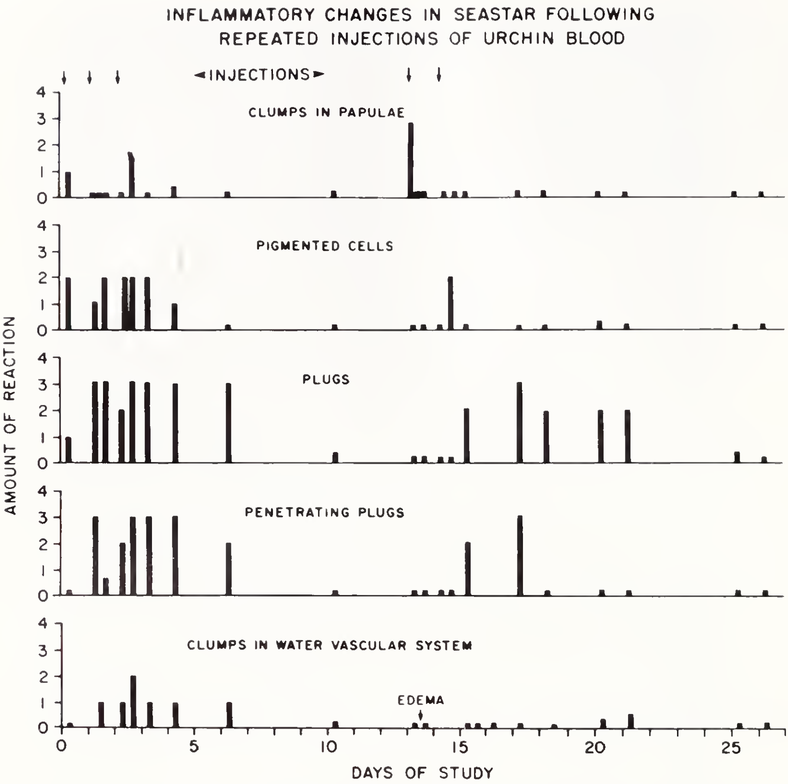

FIGURE 5. Inflammatory changes in seastar following repeated injections of urchin blood.

seastars by cell-free fluid from the injected star (Reinisch, 1974). This localization of the inflammatory response was followed by penetration of the foreign cells through the papulae, easily seen because of their bright red color. This color darkened as they were phagocytosed. They were carried through the papular tips in the same way that phagocytosed carmine particles are ejected through the tips. In order to quantify this process, five aspects of the reaction (Figs. 4 and 5) were followed in a number of small (6–10 g) seastars:

1) Clumping. The first clear sign of reaction was clumping of the urchin cells within the papulae. When 0.5 ml of freshly obtained urchin cells were injected into the arm of the seastar through a 27-gauge needle, the immediate response was minimal and usually transient; the plugs appeared later, in the papulae on both the aboral and the oral surface of the arm, usually in greater frequency on the latter.

2) Lysis. In some stars, there was a rapid (10–20 minutes) clearance of the red urchin cells (there are both red and white cells in the urchin blood, but the clearance of the colorless cells cannot be followed visually). During this rapid clearance, there was lysis of the pigmented cells. Lysis could be observed in fluid taken from injected seastars. The large red-pigmented cells rounded up, failed to send out their heavily pigmented pseudopods, individual granules became more apparent, then the granules were lysed so that the cell cytoplasm was pink; the cells finally disintegrated. In other stars, clearance required 1–2 days. We have not determined whether successive injections of urchin cells into the same seastar affect the lytic process.

3) Responses to single and multiple injections. Sequential study of individual animals showed that reaction to one injection of urchin cells was mild and usually cleared in 2–3 days (Fig. 4), that several injections were followed by greatly increased numbers of plugged papulae (Fig. 5), and that the plugged papulae would be resolved in two ways: (i) by passage through the wall of the papulae, leaving behind a hole if the mass of migrating cells was removed, and (ii) through gradual break-up within the papulae. The latter process occurred at a later time. The phenomenon was more easily followed over time in stars injected with carmine, in which circulating cells containing carmine were found as late as 4 weeks after injection.

Because of its poor adsorption to living tissue, Evans' blue dye (Noble and Gregerson, 1946) is used for determination of blood volume in mammals and for identifying localized areas of inflammation. Exposure of intact seastars for 20 minutes to a 1:1000 seawater dilution was followed by metachromatic purple staining of the feet and by blue localization wherever there had been external trauma to the skeletal spines (paxillae) and their covering epithelium. In 2 of 10 stars that were injected with urchin cells, both showed extensive diffuse staining of the aboral papulae of one limb (? the injected limb), but the other eight showed localized staining of papulae in which clumping and plugging were occurring. When 0.5 ml of this same concentration of the dye was injected into the coelomic cavity of normal stars, the dye remained within the cavity for a number of days, and several interesting events followed. During the first day, the dye gained entrance to the water vascular system but did not stain the feet purple. It was first picked up by circulating amebocytes in the papulae, then slowly by clumps of amebocytes in the feet. In addition, within one day the inner layer of the papulae stained blue and the inner and outer layer of the papulae seemed to separate, so that a cast of the inner layer became retracted from the outer wall of the papulae. By 3 days, the two epithelia rejoined and repaired.

4) Water vascular system. As stated earlier, the water vascular system is separate from the coelomic cavity. Yet some years ago we had seen, in the more transparent *Asterias rubens*, that injection of bacterial suspensions into the coelom was followed by typical tight clumping of the amebocytes within the antennae and/or feet (Bang, 1975). Clumps occurred at any place along the channel, but did not penetrate through the epithelium. Thus they could be considered a secondary result of the inflammatory process in the coelom. It is quite possible that the effect was mediated through the secretion of a neurohormonal product by the diffuse neural network. This clumping within the water vascular system was seen only occasionally following a single injection of urchin cells, but was frequently seen with the more extensive inflammation produced by several injections (Fig. 6).

5) Hemal system. The texts on echinoderm physiology refer to a third vascular system: a hemal system which seems to include the axial organ. According to Nichols (1969): "In the asteroids it is apparently not possible to distinguish a separate axial hemal system from the axial organ—the two seem to be one structure. This lies to one side of the stone canal. . . ." The similarity of the axial organ to the spleen was emphasized by Cuénot (1948), and more recently by Leclerc (1973, 1974). Millott (1966) has shown that destroyed cells are deposited in the axial organ of sea urchins. We have repeatedly dissected out this organ in the seastar following injection of foreign material, such as ciliate suspension or urchin cells, and have failed to detect any change in it. A recent paper (Kaneshiro and Karp, 1980) suggests that Tiedemann's bodies, which are outpouchings of the ring canal, may also participate in "immune reactions."

B. Other responses of seastars. 1) Edema. Edema is a well recognized com-

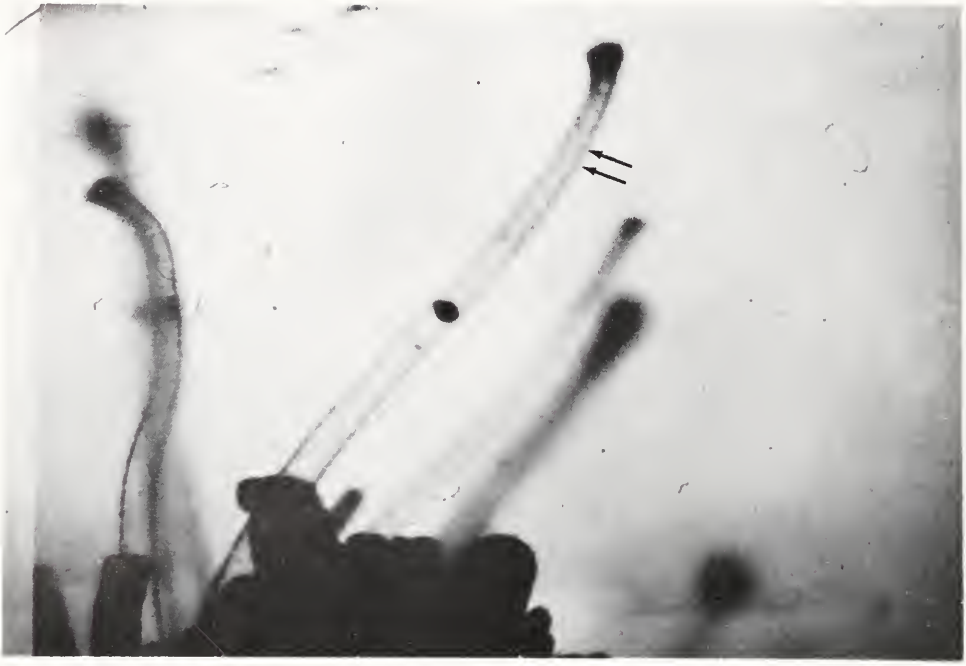

FIGURE 6. Clumps of amebocytes (see arrows) forming within extended feet of *Asterias*.

ponent of inflammation in vertebrates. It is much harder to define in invertebrates. However, an edema-like phenomenon (Bang and Lemma, 1962) did occur in this study, usually in the injected limb. From the calcareous spiny skeleton of the seastar, a series of separate spines (paxillae) project. These take a variety of forms, and along with the pedicellaria (small pincers) are covered by a thin transparent epithelium. This is usually fairly closely adherent to the skeleton and the spines, but when fluid accumulates between the skeleton and the epithelial layer, there is a ballooning of this epithelium over the paxillae so that individual spines are hidden. The response can also be easily recognized in the lateral spines on the oral surface of the individual arms. We have not been able to quantitate the response, but its gross presence was inescapable. It occurred in 5 of 14 animals in the first series, and more frequently in multiply injected animals. It occurred in the injected limb in 3 of 4 animals injected after a rest period of 11 days, and persisted for more than 48 hours.

2) Evocation of mucus-secretory substances. If this partially simplified model of inflammation is to be of any value in comparative studies, then it should mimic some of the other characteristics of vertebrate inflammmation. Inflammation of mucous membranes in vertebrates is usually accompanied by increased mucus secretion. During the last ten years, we have been interested in an invertebrate system, the urn cell complex of the coelomate *Sipunculus nudus*, which swims freely in the coelom and excretes mucus when bacterial infection occurs. These cell complexes can also be stimulated to secrete mucus *in vitro*, and we found that several mammalian sera and secretions contained mucus-stimulating substances (MSS) (Bang and Bang, 1972, 1979). In the summer of 1980, we found that seastar coelomic fluid normally contained no MSS, but that immediately following the injection of urchin cells into the seastar, a high titer of MSS appeared and persisted as long as 24 hours (Fig. 7). This was accompanied by, or perhaps caused by, the destruction of the urchin cells. It will now be of great interest to determine whether the appearance of MSS is accompanied by abnormal secretion of mucus in the seastar itself.

3) Graft rejection is commonly used by vertebrate immunologists to study immunologic reactions to various histocompatibility antigens. Graft rejection is well known among invertebrates, and the work of Théodor (1966, 1970), who showed that the branches of one sea fan are rejected by those of another, is a classic instance of recognition of self and non-self. However, much of the work on invertebrates has been unduly influenced by the standard knowledge of vertebrate immunity. Among the echinoderms, Bruslé (1967) first showed that a skin graft could be transferred and maintained for months thereafter. Ghiradella (1965) showed that the implantation of the caecum of homologous species into *Asterias* and *Patiria* was followed by retention of the implant at least 1–5 weeks, but that heterologous species (actually different genera) eliminated the foreign tissue, either through the dermal brachiae (papulae) or possibly through the host stomach. Although host amebocytes occurred in numbers around damaged tissue, much of the tissue destruction seemed to occur in their absence. Hildemann and Dix (1972) and Karp and Hildemann (1976) have studied skin graft rejection in other seastars (*Protoreaster* and *Dermasterias*) and in a sea cucumber, *Cucumaria tricolor*. They also found that control integumentary autografts remained viable but that there was

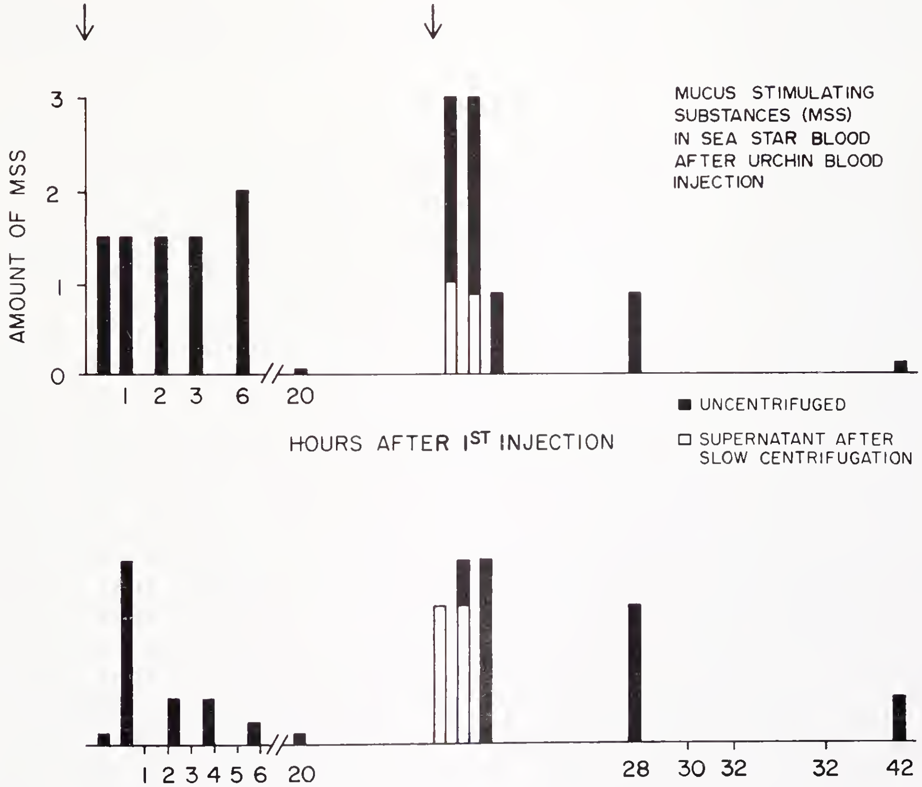

FIGURE 7. Mucus-stimulating substances (MSS) in seastar blood after urchin blood injection. Results shown represent same type experiment on two separate animals. Sea urchin blood by itself does not have mucus-stimulating activity.

slow, complete rejection of first-set heterologous grafts. To determine the specificity and memory in these graft rejections, they compared persistence in 17 first-set (213d), 5 second-set (44d), and 4 third-set (8d) grafts. Third party grafts were given to 5 animals, and 11 of these persisted longer than did the second party grafts. However, the series involved too few animals, and too little presently available histological detail, to evaluate the degree to which specificity and memory are linked, and thus to judge the possible similarity to the vertebrate system. Coffaro and Hinegardner (1977) have shown a genetic component to be important in grafting among sea urchins.

It is interesting in this regard to compare the capacity of ascidians to reject grafts by failing to fuse when grafted together. This system was extensively studied by Oka (1970) and Tanaka and Watanabe (1973). Fuke (1980) later found a counterpart reaction in which amebocytes killed each other on contact.

IV. Responses to ciliate infection

A. Background. In an early study (Bang, 1966), we found that a strong lysin against the marine ciliate *Anophrys* appeared in the serum of the marine worm *Sipunculus nudus* either during a spontaneous pox disease of *S. nudus* or after injection of foreign material into the animal. The *S. nudus* lysin was routinely effective against *Anophrys in vitro.* The sources of *Anophrys* were naturally infected *Carcinus* or *Cancer* crabs native to Roscoff in Brittany.

When seastars were injected with the blood of *Anophrys*-infected crabs, the parasite was effectively cleared from the coelom by 6 hours, and after subsequent injections was both cleared and lysed by 0.5 hours (Bang, 1975).

B. New findings. In 1981, however, we found that the rapidity of clearance and lysis of *Anophrys* after initial infection was directly related to previous exposure to marine ciliates. The first seastars used in the summer of 1981 were taken from a large stone aquarium in which a number of echinoderms were dying and were releasing a variety of marine ciliates into the running seawater. Nearly all of the seastars from this tank killed injected *Anophrys* within an hour or less. Removal of living stars to fresh running seawater for some weeks did not change this response. Only when seastars were newly collected by careful removal from mussel beds by hand, did we again routinely find animals which responded slowly.

Attempts to achieve lysis *in vitro* when *Anophrys* were added to coelomic fluid of stars that were strongly lytic *in vivo* consistently failed. This raised the question whether *in vivo* lysis was due to factors of fixed-tissue origin. The tip of an arm of a "lytic" star was cut off, carefully washed free of hepatopancreas and circulating cells, and placed in an open, plastic, minicentrifuge tube in a seawater bath. Fluid from the inside of the arm was then sampled periodically with a non-wettable plastic micropipet and placed on a slide. When *Anophrys* were added to this cell-free fluid, the ciliates were continually lysed (Bang, in press). The phenomenon was then comparatively tested and confirmed in tips of arms from clean and from other "contaminated" stars.

To summarize the lysin story: Freshly collected, previously uninjected, seastars take 6 or more hours *in vivo* to clear populations of injected *Anophrys* from the coelom. Destruction and lysis of these same ciliates occur *in vivo* within half an hour in animals injected the previous day, and in stars which are maintained under contaminated conditions. "Contamination" includes exposure to other ciliates (Grolière *et al.*, 1980), some of which infect the internal lining epithelium. *In vitro* lysis occurs in recently extirpated tips of arms that have been thoroughly washed of circulating cells, and seems to occur much more rapidly in those from "immunized" animals. The nature of the lysin, which in turn includes some autolysins, is unknown. The *Sipunculus* lysin is known to be a molecule of 250,000 daltons (Bang and Shin, 1981).

A lysin for a variety of cells is liberated from seastars by heating the stars to 75°C (Bang and Chaet, 1959), but this may be related to the presence of saponins in seastar tissue (Owellen *et al.*, 1973).

CONCLUSIONS

We now know that despite the rather limited information on natural disease processes of marine invertebrates, the sea is a rich source of disease agents, each with different potentials for diseases in different hosts. Kinne (1980) points out that the more a particular species is studied, the more agents are found. These diseases will probably increase because of the current mixing of different animals from all parts of the world, either purposefully to develop disease-resistant stock, or inadvertently by transport of local species for improving commercial yields, or for study.

Although there remain many exciting, unexplored possibilities for the study of inflammation, injury, disease and immune responses in adult seastars, we must remember Libbie Hyman's (1955) preface to her volume on the echinoderms: "I also here salute the echinoderms as a noble group especially designed to puzzle the zoologist."

ACKNOWLEDGMENT

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LIFE HISTORY OF THE CANNONBALL JELLYFISH, *STOMOLOPHUS MELEAGRIS* L. AGASSIZ, 1860 (SCYPHOZOA, RHIZOSTOMIDA)

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ABSTRACT

Stages in the life history of the scyphozoan *Stomolophus meleagris* from the planula to the newly liberated ephyra were raised in the laboratory and are described for the first time. After swimming actively for 2–5 days, the ciliated planula larvae settled and scyphistoma morphogenesis occurred. Fully developed scyphistomae were cone-shaped and bore a whorl of about 16 tentacles around a dome- or knob-shaped proboscis. Podocyst formation was the only observed method of asexual reproduction in cultures of scyphistomae maintained for one month. Strobilation began as soon as nine days after scyphistoma morphogenesis and occurred in scyphistomae with as few as eight tentacles. The strobilation process, completed in about 3.5 days at 25°C, was not accompanied by any noteworthy color changes. Most strobilae produced two ephyrae each, although the number varied from one to three. Some scyphistomae began to strobilate a second time within a week after completion of an initial round of strobilation. Newly liberated ephyrae possessed a normal complement of eight lappet pairs and eight rhopalia. They were morphologically similar to, yet distinguishable from, ephyrae of the related species *Rhopilema verrilli.* None of the examined stages of *S. meleagris* contained algal symbionts.

INTRODUCTION

Biologists did not recognize the relationship between polyp and medusa stages in the Cnidaria until life history studies were undertaken on scyphozoans by Sars (1829, 1835, 1841), Dalyell (1836), and Siebold (1839). Polypoid and medusoid forms, previously regarded as separate taxa, were both found to occur in the life cycles of *Aurelia aurita* and *Cyanea capillata.* Sars, in the course of his studies, also demonstrated that *Scyphistoma, Strobila,* and *Ephyra* were stages in the life cycle of scyphozoans rather than being separate genera.

In the years since these early studies, complete life histories have been described for few of the 200 known species of Scyphozoa. In the Hydrozoa, such investigations have become frequent to resolve problems of classification and synonymy; separate classification systems for polyps and medusae have been used in the class, and polypoid and medusoid stages of a species have often been known by different names. Comparable problems in scyphozoan systematics have been relatively minor because the conspicuous and relatively divergent species of scyphomedusae have been named and classified while their small, obscure, and morphologically indistinct polyps have not. The one notable exception is the genus *Stephanoscyphus* Allman, 1874, whose polyps are protected by a well-developed sheath of perisarc. Originally thought to be a hydrozoan, *Stephanoscyphus* gives rise to scyphomedusae of the order Coronatae.

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With the dearth of life history investigations in the Scyphozoa, stages other than the medusa are often unknown even for some of the more common species. Strobilation, a process in which the medusoid form is derived from the polypoid form, likewise remains undescribed for most scyphozoans. So it is with *Stomolophus meleagris* L. Agassiz, 1860, one of the most abundant species of scyphomedusae along the southeastern and Gulf coasts of the United States (Mayer, 1910; Kraeuter and Setzler, 1975; Burke, 1976; Calder and Hester, 1978). Various phases in the development of the medusa only have been described in this species (Mayer, 1910; Stiasny, 1922). The purpose of this paper is to describe the life history of *S. meleagris* from planula to newly liberated ephyra stages, including observations on the strobilation process.

MATERIALS AND METHODS

Stages in the life history of *Stomolophus meleagris* were raised in the laboratory from planula larvae obtained from identified medusae. Planulae were first isolated in cultures on 5 March 1974 from a holding tank containing medusae of *S. meleagris* collected in the Cooper River at Charleston, South Carolina. Scyphistomae were observed in these cultures two days later, but all died within a week. Planulae were obtained a second time from medusae collected 14 July 1981 about 1.5 km offshore from the entrance of Murrells Inlet, South Carolina. A bucket containing one male and one female medusa was placed in a mesh bag and suspended overnight just below the surface of the water. The following morning, water, debris, and planulae from the bottom of the bucket were poured into large preparation dishes. Planulae present in these dishes were isolated in a fingerbowl containing 200 ml of filtered seawater of 35.7‰ salinity. The fingerbowl was covered, and the culture was maintained at room temperature ($\approx$27°C) in the laboratory.

Developing scyphistomae were fed pieces of newly hatched *Artemia* several times a day until they were large enough to ingest entire nauplii. Undigested materials were removed daily from the cultures using a pipette, and the water was changed at least once a week. Strobilation was observed in polyps maintained both at room temperature and at 25°C in a constant temperature cabinet.

Cultures were lost, apparently due to a bacterial infection, one month after being established.

DESCRIPTIONS

Planula

Planulae, somewhat flattened in cross-section, varied in outline from elongate-cylindrical to slipper-shaped to irregularly oval (Fig. 1a) and measured 120–390 μm long and 60–130 μm wide. The anterior and posterior ends were rounded. They were mouthless, and a solid, inner, endodermal mass was enclosed by an outer, ciliated ectoderm. Living planulae were translucent-whitish in color.

Scyphistoma

At metamorphosis, planulae attached by the anterior end to the substrate and gradually became flask-shaped as the narrowing stalk of the developing scyphistoma differentiated from the expanding calyx. Newly metamorphosed scyphistomae (Figs. 1b,c) varied from 200–430 μm in height from pedal disk to mouth. A thin cuticle enveloped the slender stalk. Tentacles, usually four in number but sometimes

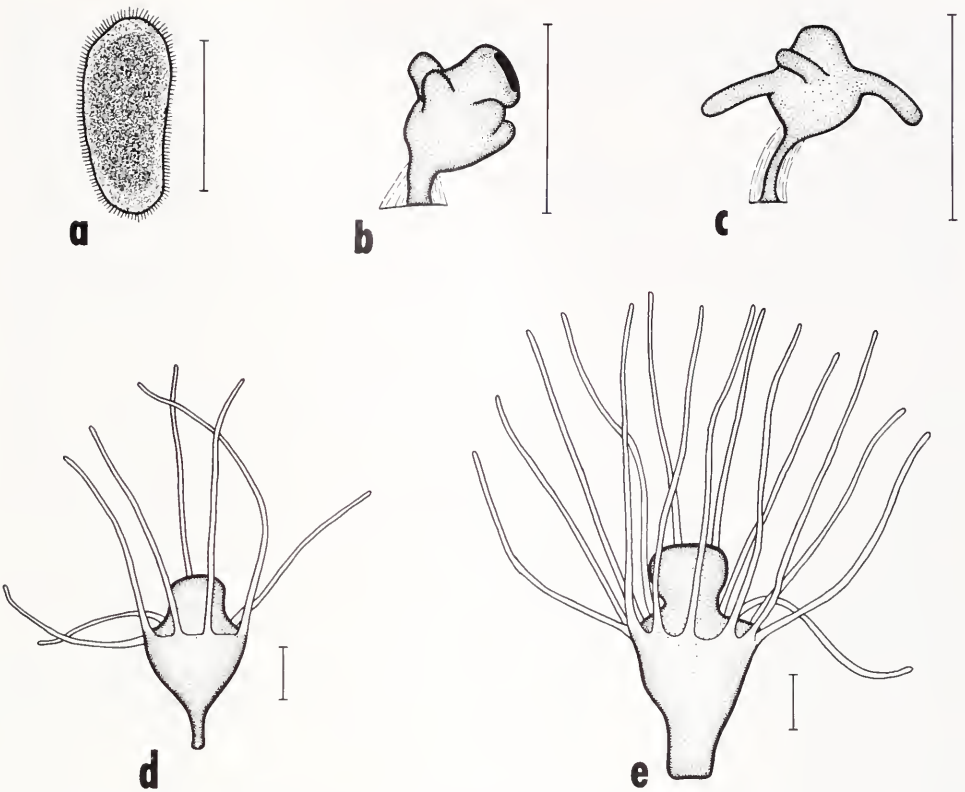

FIGURE 1. Planula and scyphistoma stages of *Stomolophus meleagris*. a. Planula. b. Newly metamorphosed scyphistoma. c. Young scyphistoma. d. Intermediate, 8-tentacled scyphistoma. e. Fully developed scyphistoma. Scale bars = 250 μm.

as few as two and as many as six, appeared near the distal end of the bulbous calyx. They were filiform with scattered nematocyst batteries, and occurred in one whorl. The oral disk was largely occupied by a prominent, dome-shaped proboscis. The mouth, round or irregular in shape, was capable of considerable dilation. The color was translucent-whitish.

Intermediate scyphistomae (Fig. 1d) were 0.5–1.0 mm high and bore eight very contractile tentacles. These were filiform with scattered nematocyst batteries, and occurred in one whorl. The stalk was slender, and the cuticle originally enclosing the stalk had become vestigial. The calyx was cone-shaped. A large, flexible, dome-shaped proboscis occupied most of the oral disk, which bore four peristomial pits. The expansible mouth was round, irregular, or quadrate, and the pharynx was quadrate. The color was translucent-whitish, but the endoderm became light orange after digestion of *Artemia*.

Fully developed scyphistomae (Fig. 1e) attained about 2 mm in height from pedal disk to mouth and bore about 16 filiform tentacles. Tentacles were contractile, in one whorl, and had scattered nematocyst batteries. The moderately thick stalk was somewhat variable in length and merged almost imperceptibly with the cone-shaped calyx. The proboscis was large, flexible, and dome- or knob-shaped. The mouth continued to be expansible, and was round, irregular, or quadrate in shape.

The pharynx was quadrate, and the oral disk bore four prominent peristomial pits. The color was whitish, with the endoderm becoming light orange after digestion of *Artemia.*

Strobila

Elongation of the calyx occurred in the early strobila, but the first clear external indication of strobilation was the development of a small marginal lobe at the base of each rhopalar tentacle (Fig. 2a). About six hours later, segmentation began as a faint circular incision proximal to the tentacular ring; this incision became progressively deeper and more pronounced (Fig. 2b). A second or even a third incision

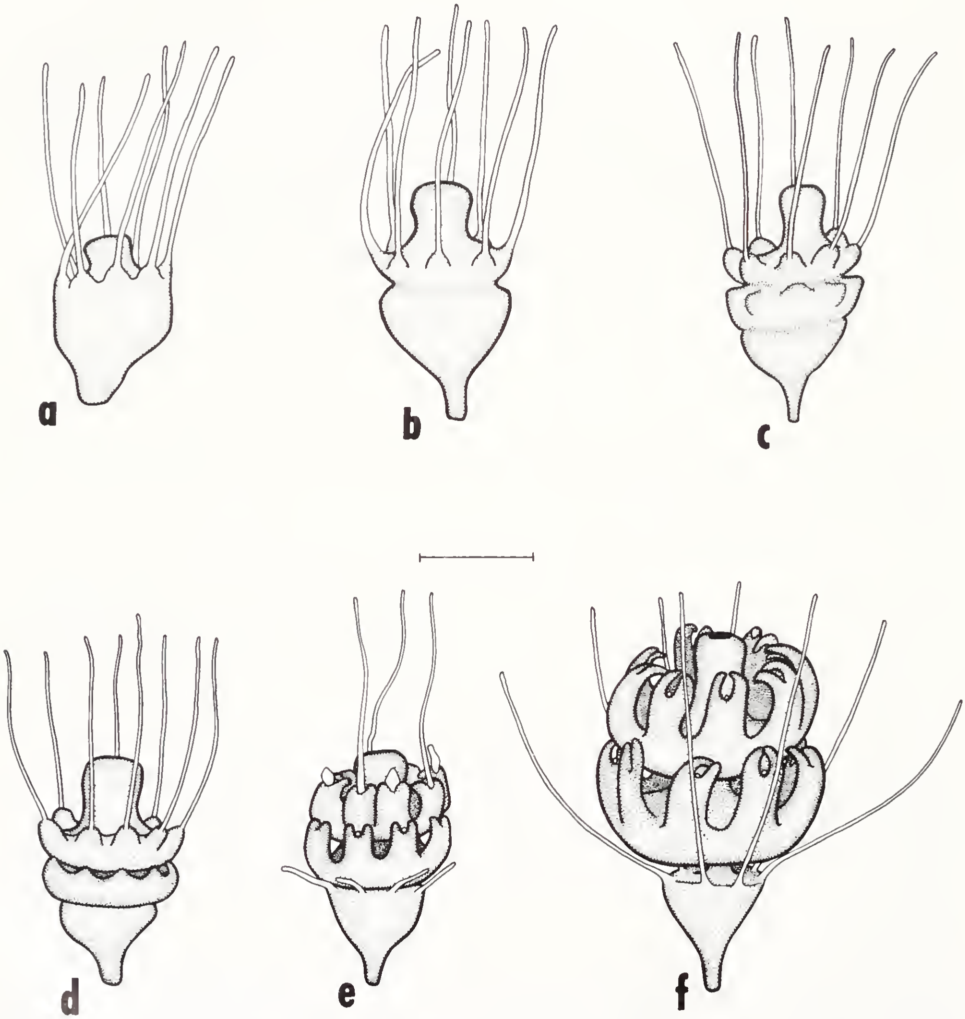

FIGURE 2. Strobilae of *Stomolophus meleagris.* a. Early strobila with tentacular lobes. b. Early strobila with incision. c. Early strobila with second incision. d. Early strobila with developing segments. e. Mid-strobila, with regressing tentacles and developing ephyral segments. f. Late strobila with well developed ephyra segments and basal polyp. Scale bar = 500 μm.

subsequently appeared proximal to the first in polydisk strobilae, each forming a segment representing an incipient ephyra (Fig. 2c). As strobilation proceeded, the proboscis became enlarged, tentacular lobes on the distal segment became more pronounced, and marginal lobes became progressively better defined about the periphery of any proximal segments (Figs. 2c,d).

About 36 hours into the strobilation process, tentacles began to undergo regression, contracting and expanding periodically. The eight rhopalar tentacles were resorbed, some more rapidly than others (Fig. 2e), into the cleft between the developing lappets on each marginal lobe. If tentacles in addition to the eight rhopalar ones were present, they were resorbed into the ocular clefts between the marginal lobes. Simultaneous with tentacular regression, rhopalia with statoliths became apparent, lappets elongated, incisions constricting the segments deepened, manubrium development progressed on any proximal ephyral segments, a new proboscis began to form on the basal polyp, and the polyp began to regenerate new tentacles. Nascent ephyrae, beginning with the one at the distal end, became capable of contractions. These movements were weak at first, but became progressively stronger and more frequent. Tentacles of the original scyphistoma were completely resorbed about 54 hours into strobilation.

In the late strobila (Fig. 2f), incisions continued to deepen and separate the developing ephyrae. Ephyrae increased markedly in size and underwent rapid development prior to release. Pulsations became stronger and occurred more frequently. Development of the manubria in proximal ephyrae and proboscis in the polyp proceeded. Gastric cirri were visible in the developing ephyra prior to liberation. Ephyrae were liberated about 3.5 days after strobilation began.

Ephyra

Newly liberated ephyrae (Figs. 3a,b) were about 1.5–2.0 mm wide from lappet-tip to lappet-tip. There were typically eight marginal lobes, eight rhopalia, and eight pairs of slender, distally pointed lappets. Rhopalar clefts were U-shaped and slightly more than half as deep as the large ocular clefts separating the marginal lobes. The manubrium was small and cruciform in cross-section. Four lips were often present about the mouth, but oral arms were lacking and no papillae were present. The stomach portion of the gastrovascular cavity was nearly circular, and 1–2 gastric cirri were present in each quadrant. Eight blunt-ended rhopalar canals and eight small adradial bulges extended peripherally from the central stomach. Coronal muscles were barely evident in unstained specimens, but the radial muscles were visible extending to each lappet from the marginal lobes. The exumbrella was marked by a ring of small nematocyst batteries about the periphery of the stomach, and a large, elongate battery was present on each marginal lobe. The mesoglea was thin. The ectoderm was pale straw colored, while the remainder of the ephyra was translucent.

General Observations

Planula larvae of *S. meleagris* are apparently not incubated by the adult medusae. Medusae of both sexes were dissected and examined microscopically for incubated planulae during studies in 1974 and 1981, but none were found. Planulae obtained both years were found free in containers of water in which sexually mature medusae had been held overnight.

Planulae swam actively by ciliary propulsion, rotating counterclockwise around the anterior-posterior axis. Within two to five days they attached by the anterior

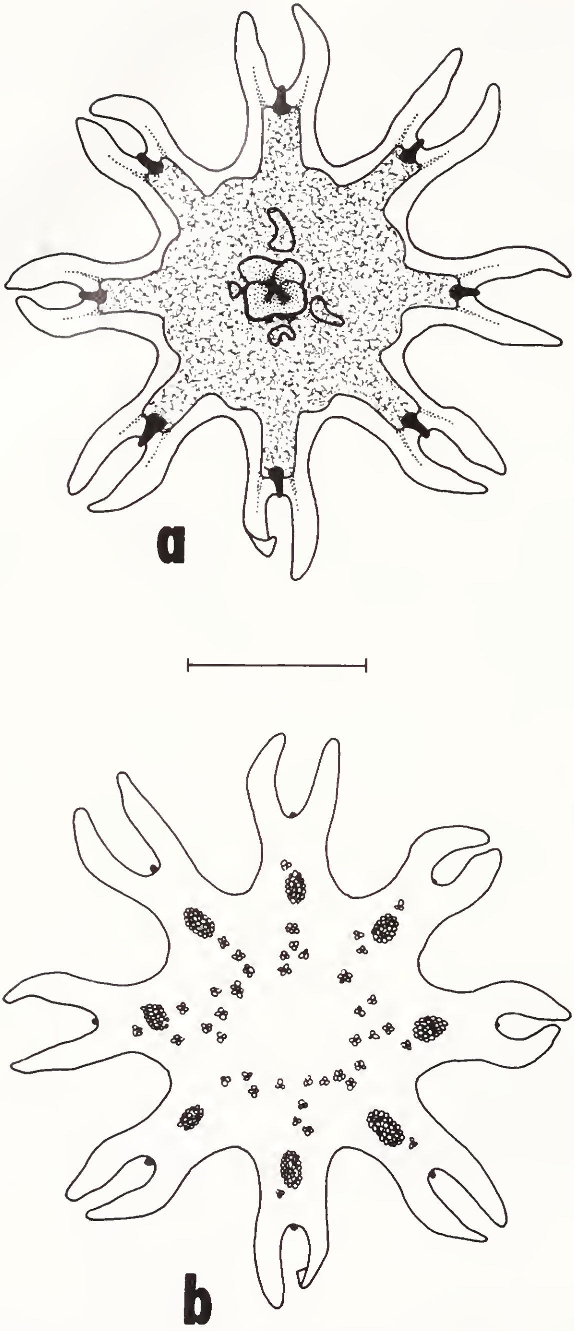

FIGURE 3. Newly liberated ephyra of *Stomolophus meleagris.* a. Subumbrellar view. b. Exumbrellar view. Scale bar = 500 μm.

end and metamorphosed directly into scyphistomae, without an intervening larval cyst stage.

Recently metamorphosed scyphistomae were sessile, but if dislodged were capable of slow locomotion along the bottom of the container using the cilia. Dislodged polyps eventually reattached through the formation of a pedal stolon, which became anchored to the substrate. Little is known about asexual reproduction in the polyp of *S. meleagris*. However, one scyphistoma with 16 tentacles was forming a podocyst shortly before loss of the culture.

Strobilae were first observed in the cultures only nine days after polyp morphogenesis occurred. Scyphistomae as small as 1 mm or less, and bearing as few as eight tentacles, became strobilae. The strobilation process was neither preceded nor accompanied by any noteworthy changes from the translucent, pale straw color assumed by the developing ephyrae. In most cases, two ephyrae were formed by each strobila, although as few as one and as many as three were produced on occasion. Some of the scyphistomae of *S. meleagris* strobilated a second time, as soon as a week after completion of the initial strobilation.

Ephyrae produced during strobilation swam actively and appeared to be normal morphologically. No attempt was made to raise the ephyrae, and most were preserved shortly after liberation.

None of the life stages of *S. meleagris* examined during this study bore algal symbionts.

Discussion

Agassiz (1860, 1862) described and illustrated the medusa of *Stomolophus meleagris* from specimens collected at Wassaw Island, Georgia, and Charleston, South Carolina. The species has subsequently been reported from New England to Brazil in the western Atlantic (Kramp, 1961; Larson, 1976), but its occurrence north of Cape Hatteras is probably due largely or entirely to transport in water currents. Elsewhere, it has been recorded from southern California to Ecuador in the eastern Pacific and from the Sea of Japan to the South China Sea in the western Pacific (Kramp, 1961; Omori, 1978). In the orient, *S. meleagris* is one of several rhizostome medusae used for human consumption (Omori, 1978, 1981).

Medusae of *S. meleagris* are frequent in coastal waters from North Carolina to Florida and in the northern Gulf of Mexico (Mayer, 1910). Kraeuter and Setzler (1975) reported finding medusae of this species from March through October in Georgia, and Burke (1976) noted that *S. meleagris* was almost always present in Mississippi Sound. In South Carolina, they occur sporadically throughout the year and at times are a hinderance to commercial shrimp trawling because of their abundance (Calder and Hester, 1978). As with many species of scyphomedusae, abundances vary greatly from year to year as well as from season to season. Despite their frequency of occurrence in waters of the southeast and Gulf coasts of the United States, medusae of this species are an insignificant public health hazard because the toxin of their nematocysts is relatively innocuous to humans (Toom and Chan, 1972).

The life history of *S. meleagris* (Fig. 4) resembles that described for other species of neritic Scyphozoa (Naumov, 1961; Russell, 1970). The fertilized egg develops into a tiny, motile planula larva. After swimming freely in the water for several days, the planula attaches to a suitable substrate and transforms into a sessile polyp or scyphistoma. Scyphistomae feed and grow, attaining a maximum size of a few millimeters. They reproduce asexually a number of ways, including the formation of podocysts and motile or non-motile buds, but only podocyst formation was observed in *S. meleagris*. In addition to their function in asexual reproduction, podocysts in the Scyphozoa are resistant to adverse environmental conditions (Cargo and Schultz, 1966). Under favorable conditions the scyphistoma

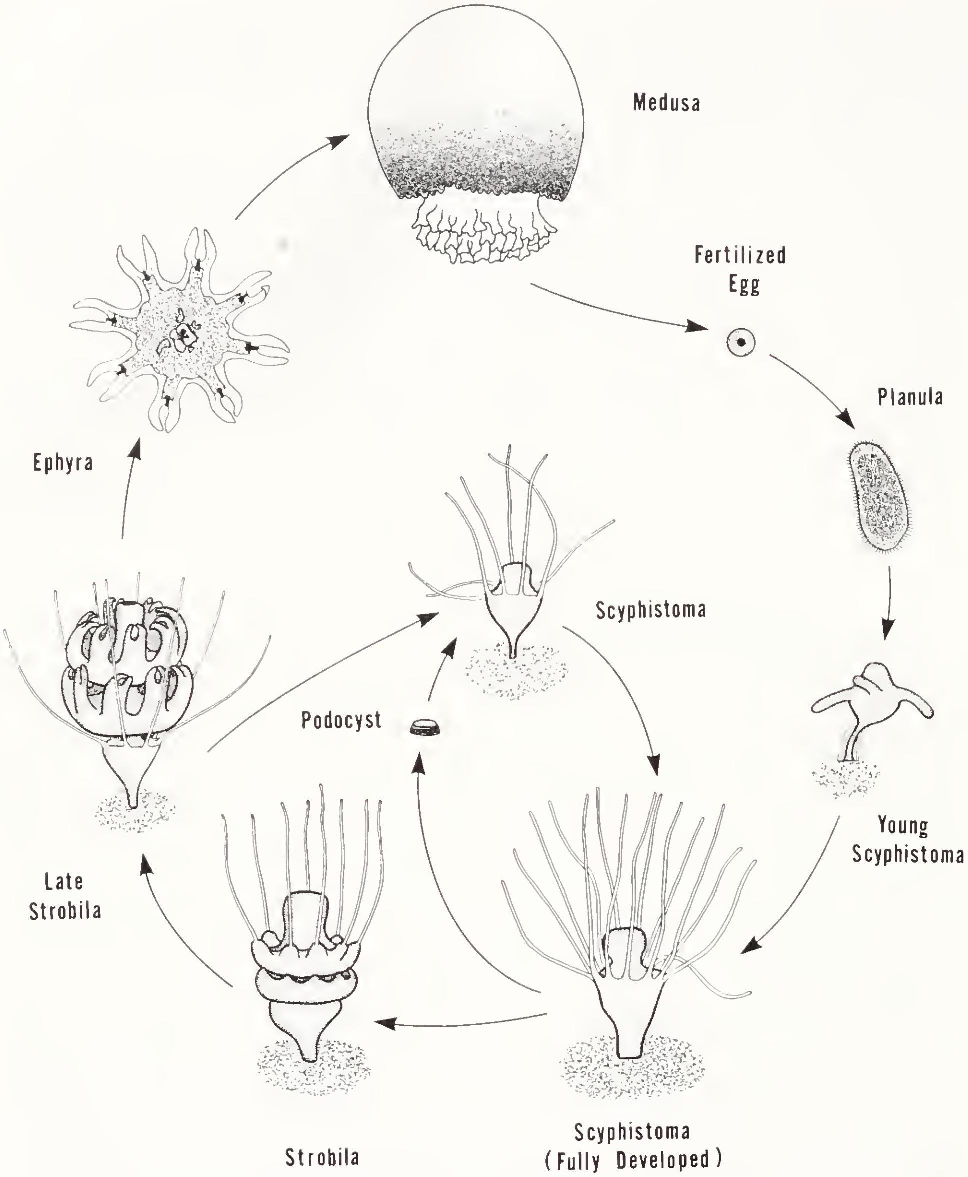
Medusa
Fertilized Egg
Planula
Young Scyphistoma
Scyphistoma (Fully Developed)
Strobila
Late Strobila
Ephyra
Scyphistoma
Podocyst

FIGURE 4. General life history of the scyphozoan *Stomolophus meleagris*. See text for explanation.

undergoes strobilation; during this process the polyp is known as a strobila. Two separate developmental phenomena, namely segmentation and metamorphosis, are involved in the strobilation process (Thiel, 1938; Spangenberg, 1968). Strobilation results in the derivation of several organisms from a single individual. One or more free-swimming ephyrae may be produced, and the basal portion of the strobila is left as a small scyphistoma after release of the ephyrae. This small scyphistoma rapidly returns to normal size and is capable of repeated strobilation. Ephyrae develop into medusae, completing the life cycle.

Scyphopolyps have been described for relatively few species of Scyphozoa. Known polyps of the order Coronatae are distinctive in being elongate forms enveloped in a chitinous tube (Russell, 1970). They resemble, and were believed by Chapman (1966) and Werner (1966, 1967a,b, 1970, 1973) to be related to, the fossil Conulata. Chapman (1973) observed behavioral as well as morphological differences between coronate and semaeostome polyps. Semaeostome and rhizostome scyphistomae display considerable morphological similarity from species to species within and between orders. The basic form is that of a goblet or cone, attached aborally by a pedal disk, and bearing an oral whorl of tentacles (Russell, 1970). A cuticular theca, if present at all, is vestigial (Chapman, 1966, 1968).

Among the rhizostomes, scyphistomae are known for *Cassiopea andromeda, Cephea cephea, Cotylorhiza tuberculata, Mastigias papua, Rhizostoma pulmo, Rhopilema verrilli*, and now *Stomolophus meleagris* (Table I). Of these, *S. meleagris* most closely resembles *R. verrilli* in its cone-shaped calyx and long proboscis. The only reliable way to distinguish polyps of these two species at present would be to induce and observe strobilation. Red and orange pigments appear during strobilation in the developing ephyrae of *R. verrilli* that are lacking in *S. meleagris*. Studies on the nematocysts of scyphistomae and other stages of *S. meleagris*, underway as part of another investigation, may provide a means of separating the two species. Diagnostic differences in the nematocyst complement were detected in scyphistomae of the semaeostomes *Aurelia aurita, Chrysaora quinquecirrha*, and *Cyanea capillata* (Calder, 1971).

Scyphistomae of *S. meleagris* began to strobilate prior to attaining full development. Polyps as young as nine days old, measuring 1 mm or less in height and bearing as few as eight tentacles, were observed undergoing strobilation. Ephyrae produced by these strobilae appeared normal in all respects. Strobilation so soon after scyphistoma morphogenesis was unexpected but may not be unusual in the Scyphozoa, given optimal conditions. Strobilation has also been observed, under field conditions, in polyps of *Chrysaora quinquecirrha* that were less than two weeks old (D. G. Cargo, Chesapeake Biological Laboratory, personal communication).

Strobilation in the Scyphozoa may be monodisk, in which one ephyra is produced, or polydisk, in which two or more ephyrae are produced. Although monodisk strobilation appears to be common in the rhizostomes (Table I), it can no longer be regarded as a fixed characteristic of the group, as suggested by Sugiura (1966). Polydisk strobilation was the norm in *S. meleagris*, and has been observed in three other species of rhizostomes (Table I). Conversely, Uchida and Sugiura (1978) demonstrated that strobilation in the semaeostome *Sanderia malayensis* was monodisk. Strobilation in semaeostomes such as *Cyanea capillata, Aurelia aurita*, and *Chrysaora quinquecirrha* is usually, but not always, of the polydisk type (Berrill, 1949; Spangenberg, 1968; Calder, 1974). Berrill (1949) concluded that the type of strobilation depended upon the size and shape of the scyphistoma. Monodisk strobilation usually occurs in polyps having a short calyx, while polydisk strobilation normally occurs in polyps having an elongate, columnar calyx. The type of strobilation thus appears to have little or no phylogenetic significance within the Scyphozoa.

Several polyps underwent strobilation twice during the month that cultures of *S. meleagris* were maintained in the laboratory. However, strobilation several times in succession is well known in the Scyphozoa, having been reported in species such as *Chrysaora quinquecirrha* (Cargo and Schultz, 1967; Loeb, 1972; Calder, 1974; Cargo and Rabenold, 1980), *Aurelia aurita* (Thiel, 1962), *Cassiopea andromeda*

TABLE I

A summary of some attributes of seven known species of rhizostome polyps.

Species	Shape	No. tentacles	Color	Algal symbionts	Asexual reproduction	Strobilation	Strobila color	References
Cassiopea andromeda (Forskål, 1775)*	goblet-shaped, stalk long, oral disk wide	32±	greenish brown	present (sometimes absent in young scyphistomae)	motile buds	monodisk (infrequently polydisk)	greenish brown	Bigelow (1900) Gohar & Eisawy (1960b) Ludwig (1969) Neumann (1977, 1979) Hofmann, *et al.* (1978) Neumann, *et al.* (1980) Rahat & Adar (1980)
Cephea cephea (Forskål, 1775)	goblet-shaped, stalk long, oral disk width moderate	16	white	absent	motile buds	monodisk	yellow, yellowish brown	Sugiura (1966)
Cotylorhiza tuberculata (Macri, 1778)	goblet-shaped, stalk long, oral disk width moderate	16		present	motile buds	monodisk		Claus (1890, 1893)
Mastigias papua (Lesson, 1830)	goblet-shaped, stalk long, oral disk width moderate	16	white	absent (zooxanthellae could be introduced)	motile buds	monodisk	yellow-green	Uchida (1926) Sugiura (1963)

TABLE I—*Continued*

Species	Shape	No. tentacles	Color	Algal symbionts	Asexual reproduction	Strobilation	Strobila color	References
Rhizostoma pulmo (Macri, 1778)	cone-shaped, stalk length moderate, oral disk wide	32		absent	motile buds, polyp buds, stolon buds, podocysts, strobila buds	polydisk		Paspaleff (1938)
Rhopilema verrilli (Fewkes, 1887)	cone-shaped, stalk length moderate, oral disk width moderate, proboscis clavate	16+	whitish	absent	podocysts	monodisk (occasionally polydisk)	whitish, developing ephyrae with red and orange pigments	Cargo (1971) Calder (1973)
Stomolophus meleagris L. Agassiz, 1860	cone-shaped, stalk length moderate, oral disk width moderate, proboscis dome- or knob-shaped	16	whitish	absent	podocysts	polydisk (occasionally monodisk)	whitish	

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* Gohar and Eisawy (1960a) and Neumann (1979) have been followed in regarding *Cassiopea xamachana* Bigelow, 1892 as a synonym of *C. andromeda* (Forskål, 1775).

(Gohar and Eisawy, 1960a), *Mastigias papua* (Sugiura, 1963), and *Cephea cephea* (Sugiura, 1966).

The ephyra is the least differentiated and most difficult stage to identify in the development of the medusa of scyphozoans. Russell (1970) distinguished ephyrae of *Aurelia aurita, Chrysaora hysoscella, Cyanea* spp., and *Rhizostoma octopus* from Britain using morphological differences in radial canal shape, number of gastric filaments, marginal tentacle development, and arrangement of nematocyst batteries on the exumbrella. Ephyrae of *Aurelia aurita, Chrysaora quinquecirrha, Cyanea capillata*, and *Rhopilema verrilli* from the east coast of the United States have been distinguished on the basis of morphology (Larson, 1976) and nematocyst complement (Calder, 1977). Of these four species, newly liberated ephyrae of *S. meleagris* most closely resemble *R. verrilli*. Unlike *R. verrilli*, they have (1) faint instead of prominent adradial bulges in the gastrovascular cavity; (2) a small manubrium with no papillae; (3) no rose or orange pigments in the stomach and manubrium endoderm; (4) a different arrangement of nematocyst batteries on the exumbrella. Ephyrae of *S. meleagris* were also smaller at liberation than those of *R. verrilli* described by Calder (1973), but this may have been due in part to the small size of the strobilae.

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SPECIFICITY OF THE HOST-INDUCED NEGATIVE PHOTOTAXIS OF THE SYMBIOTIC WATER MITE, *UNIONICOLA FORMOSA*[1]

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ABSTRACT

The water mite *Unionicola formosa* (Acarina: Unionicolidae) exhibits positive phototaxis when free of any chemical influence of its molluscan host, *Anodonta imbecilis*. When mites are exposed either to water from the host's mantle cavity or to an homogenate of host mantle tissue, the sign of their phototaxis reverses to negative. The behavioral threshold concentration of mantle homogenate that induces negative phototaxis to monochromatic light is approximately 0.7 μg protein/ml. Negative phototaxis does not occur in the presence of any chemical influence of the bivalves *Elliptio complanata* or *Anodonta cataracta*, neither of which species harbors this symbiont at the study site. A component of mantle tissue from *Anodonta imbecilis* that elicits the negative phototaxis is heat labile, sensitive to trypsin, and has a molecular weight in excess of 10,000 daltons.

INTRODUCTION

Various mechanisms of communication involving a variety of sensory modalities are instrumental in the interactions between species associated in symbiotic relationships in marine and freshwater ecosystems (Davenport, 1966; Ache, 1974; Fricke, 1975). Chemical communication, often highly specific, consistently achieves prominence in the initiation and maintenance of such interspecific associations (Davenport, 1955; McCauley, 1969; Mackie and Grant, 1974; Barbier, 1981).

Unionid bivalve molluscs frequently serve as hosts for symbiotic freshwater mites of the genus *Unionicola* (Acarina: Unionicolidae) (Mitchell, 1955; Davids, 1973; Hevers, 1980; Vidrine, 1980). Presumably, the host's mantle cavity could be a refuge from predators or perhaps environmental stresses, while the water circulated through the mantle cavity may convey food to the symbionts. The host itself can be exploited as a source of nutrition (Baker, 1977) or as a site for oviposition and metamorphosis (Mitchell, 1955).

The specificity of these molluscan-acarine symbioses may be influenced by different aspects of the biology of the organisms involved in them, such as behavior, population dynamics and distributional patterns. For example *Unionicola formosa* is widely distributed in eastern North America (Vidrine, 1980) and throughout its range exhibits varying degrees of host specificity as revealed by field data (Dobson, 1966; Roberts, 1977; Gordon *et al.* 1979; Vidrine, 1980). Specificity has also been demonstrated in the host recognition behavior of *U. formosa* (LaRochelle and Dimock, 1981). In addition Welsh (1930) reported that *U. ypsilophora* (probably

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Abbreviations: APW, artificial pond water; MH, mantle homogenate.

U. formosa, see Roberts *et al.*, 1978) reversed completely its normally positive phototaxis when tested both in water from the mantle cavity and in gill extract of its host, the freshwater mussel *Anodonta cataracta.* Subsequently Welsh (1931) indicated that this behavior could only be induced in the presence of the mite's own molluscan host, *i.e.*, the host-induced negative phototaxis was species-specific with respect to its induction.

With the exception of the observations of Welsh (1930, 1931) there has been no further study of the factors affecting the host-induced negative phototaxis of *U. formosa* except for an analysis of the spectral sensitivity of the response (Roberts *et al.*, 1978). The present paper quantifies the dose-response relationships of the negative phototaxis of *Unionicola formosa* induced by a tissue homogenate of its molluscan host in the southeastern U. S., *Anodonta imbecilis*, and examines the species-specificity of the induction of this behavior. Results of initial chemical characterization of the substance of host origin that elicits the negative phototaxis of *U. formosa* are also presented.

Materials and Methods

Organisms

With the exception of *Elliptio complanata* which was purchased from Carolina Biological Supply Co., the animals used in this study were collected as needed from the farm pond of Mr. James Honeycut, Mt. Pleasant, Cabarrus County, North Carolina, where *Anodonta imbecilis* is sympatric with *Anodonta cataracta.* Because the density of female *U. formosa* in host bivalves increases in a linear fashion with increasing bivalve length (Dimock, 1979; Gordon *et al.*, 1979), only mussels > 60 mm in total length were used in the experiments. Since female *U. formosa* are far more numerous than males (Dimock, 1979), only females were used.

All *U. formosa* employed in this study were collected from *A. imbecilis.* Mites removed from host mussels were first held for at least 24 h in artificial pond water (APW) (Dietz and Alvarado, 1970). They were then transferred twice to fresh APW for 1 h, after which they were rinsed in 2 changes of APW immediately before use in the phototaxis assay. All mites were used within 36 h of isolation from their host.

Mantle homogenate (MH)

The precise standardization of a chemical stimulus for behavioral studies, although highly desirable, is impossible if one is working with a crude homogenate of a tissue. All tissue homogenates employed in the present study were, therefore, quantified on the basis of protein concentration as determined by the Bradford technique (Bradford, 1976) standardized with bovine serum albumin (Sigma Chemical Co.). In order to avoid potential contamination with *U. formosa's* eggs which are deposited in the host's gills (Vidrine, 1980), only mantle tissue of the mussels was used to prepare experimental homogenates [Mantle Homogenate (MH)].

MH was prepared by macerating fresh mantle in an iced tissue homogenizer with APW and then centrifuging at 48,000 × *g* at 4°C for 2 hours. The supernatant was frozen at −80°C in microcentrifuge tubes (0.5 ml, Brinkmann) until it was used, since preliminary experiments had revealed that the effect of MH on phototaxis is not altered by freezing and storage for 30 days.

Phototaxis

Phototaxis was monitored in a 120 × 20 × 20 mm chamber constructed of black lucite (except for transparent ends) which was provided with a removable partition that subdivided the chamber into five 22 mm compartments. The chamber was illuminated horizontally from one end with a 100 W tungsten filament spot photographic light that was filtered first through an infrared filter (Corning #1-75) and a "hot-mirror" (Baird Atomic) to reduce the radiation above 700 nm. The light intensity (measured at the chamber) and wavelength were then filtered (Ditric Optics Co.) to 10^{-1} $\mu E/m^2/sec$ and 500 nm, the optimum light stimulus for negative phototaxis (Roberts *et al.*, 1978). Light intensity was measured with a Lambda Instrument Corp. Quantum Sensor (Model LI-185).

A typical test involved dark-adapting 30 mites in 1 ml of APW in a 12 × 75 mm plastic tube (Falcon 2003) for 1.5 h at 20°C. The mites were then poured in the dark into the center section of the chamber which had previously been filled with 29 ml of the desired test medium also at 20°C. After 30 seconds the stimulus light was turned on and the partition was removed for 90 seconds; it was then reinserted and the number of mites in each section was recorded.

Where appropriate the data were analyzed by the Kolmogorov-Smirnov goodness of fit test (Zar, 1974), one-way ANOVA, and Student Newman Kuels (SNK) test. The data are presented as percent response based on the number of mites in the section of the chamber closest to the light source (positive phototaxis) or the section farthest from the light source (negative phototaxis). Every experiment was repeated three times with different mites in each replicate for a total of 90 animals per experiment.

Dose-Response

Concentrations of MH (0.2 to 30 μg of protein/ml) prepared from *A. imbecilis* were tested in the chamber for their effects on the phototaxis of *U. formosa*.

Host specificity

The specificity of the induction of negative phototaxis was tested by comparing the responses of *U. formosa* to the mussels *Anodonta imbecilis*, *Anodonta cataracta*, and *Elliptio complanata*. Two different experimental approaches were employed. In the first, 12 live mussels of each species were scrubbed in APW to remove any debris from the shells and the water from the mantle cavity was gently removed. Each species was then placed in a clean aquarium with 2000 ml of APW for 38 h, after which 30 ml of water was obtained from the mantle cavities and used as the test medium. In the second approach, MH of each of the three species was prepared as described above and tested at a concentration of 6.67 μg protein/ml.

Ultrafiltration and heat sensitivity

Fifteen ml (30 mg protein) of MH of *A. imbecilis* was ultrafiltered to 1 ml with an Immersible Cx-10 ultrafilter (nominal moleclular weight exclusion of 10,000 daltons, Millepore Corp.). The low ($<10^4$ daltons) molecular weight fraction, which contained no detectable protein, was subdivided into three aliquots which were each made up to 30 ml with APW and tested for their effect on phototaxis. The high molecular weight fraction was tested at 6.67 μg protein/ml.

Unfractionated MH was heated at 60, 80 and 100°C for 30 min in a water bath and each solution was then bioassayed.

Trypsinization

The effect of digestion by trypsin on the potential of MH from *A. imbecilis* to induce negative phototaxis was examined by treating MH (7 mg protein) with 10 μg of trypsin (Difco) in a total volume of 1.0 ml for 2 h at 25°C. From this solution 30 μl aliquots were bioassayed in a final volume of 30 ml (MH = 6.67 μg protein/ml; trypsin = 0.01 μg/ml).

Control experiments tested the effects of exposure to trypsin on the positive and negative phototaxis of *U. formosa.* Mites were tested for the sign and magnitude of their photobehavior in APW with 0.01 μg trypsin/ml. Also, mites that had been incubated for 1 h in 10 μg trypsin/ml were tested in MH (6.67 μg protein/ml) to determine if trypsin affected the mite's detection of the chemical signal.

RESULTS

Phototaxis

The distribution of mites among the five compartments of the test chamber when held for 90 seconds in total darkness in APW with or without mantle homogenate was not significantly different from an expected distribution of 20% of the animals in each compartment (Kolmogorov-Smirnov; $P > 0.05$). However, their distribution in response to the stimulus light in APW was highly significantly different (Kolmogorov-Smirnov; $P < 0.01$; $\bar{X}$% positive phototaxis = 87%) from that in the dark and clearly substantiated their positive phototaxis when tested in APW free of any chemical influence of *A. imbecilis.* The phototactic response of *U. formosa*, however, was significantly negative (Kolmogorov-Smirnov; $P < 0.01$; $\bar{X}$% negative phototaxis = 50%) when the animals were exposed to the stimulus light both in water from the mantle cavity of *A. imbecilis* and in supra-threshold concentrations of MH of *A. imbecilis.*

Dose-response

The results of the dose-response experiments (Fig. 1) revealed that MH at a concentration of 0.83 μg protein/ml was an adequate stimulus to induce negative phototaxis. Furthermore, while all concentrations of MH > 0.83 μg protein/ml induced responses that were significantly different from the responses to concentrations lower than that dose (ANOVA; SNK; $P < 0.05$), the responses of *U. formosa* to all concentrations > 0.83 μg protein/ml were not significantly different from each other (ANOVA; $P > 0.05$).

Host specificity

The sign and magnitude of the phototaxis of *U. formosa* in the presence of MH from various species of mussels are depicted in Figure 2. It is obvious that only MH from *A. imbecilis* induced negative phototaxis by the mites. This marked specificity also occurred when *U. formosa* was tested for its sign of phototaxis in water from the mantle cavities of these three species of mussels. In those experiments water from *A. imbecilis* induced 40% negative and 4% positive phototaxis; that from *A. cataracta*, 4% negative and 83% positive phototaxis; and that from *E. complanata*, 8% negative and 82% positive phototaxis.

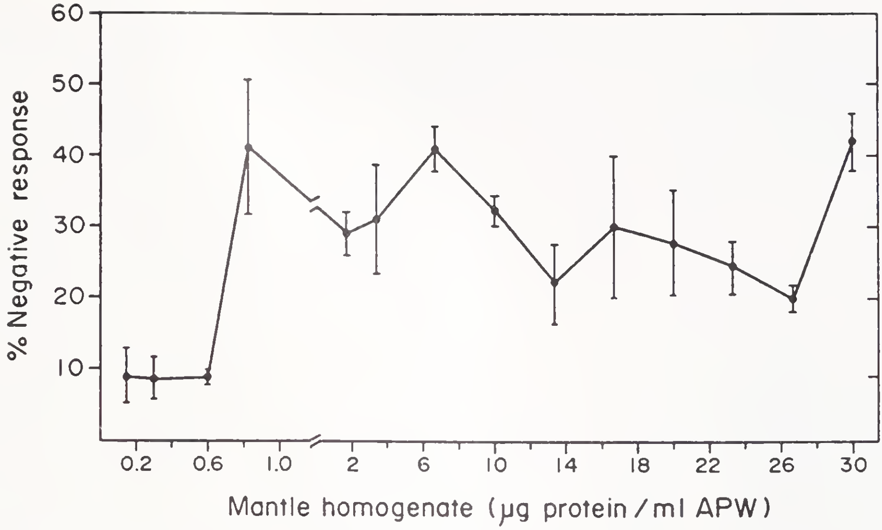

FIGURE 1. Dose-response relationships of the negative phototaxis of *Unionicola formosa* induced by mantle homogenate of *Anodonta imbecilis*. Data points are the $\bar{X} \pm$ SE (N = 90). All concentrations of mantle homogenate $\geqslant 0.83$ μg protein/ml elicited negative phototactic responses that were not different from each other (ANOVA, $P > 0.05$) but that all were significantly greater than the responses to lower concentrations (ANOVA; SNK; $P < 0.05$).

Characterization of mantle homogenate

Only the high molecular weight fraction ($>10^4$ daltons) of ultrafiltered MH elicited negative phototaxis (Table I). The magnitude of the negative response induced by this fraction was significantly higher ($P < 0.05$, t test) than that elicited by unmodified homogenate.

Heating MH at 80°C and at 100°C for 30 minutes destroyed its capacity to induce negative phototaxis (Table I). Although heating MH at 60°C for 30 min resulted in a significant reduction in the negative response (and a concomitant increase in positive phototaxis), the potential of MH to induce negative phototaxis was not completely eliminated by this milder heat treatment (Table I).

Trypsinization of MH resulted in a significant reduction of the magnitude of negative phototaxis and restored the level of positive phototaxis to about 60% of that which occurs in plain APW (Table I). Exposure of mites to trypsin in the absence of MH had no significant effect on their positive phototaxis. In addition, the incubation of *U. formosa* for 1 h in a 1000-fold higher concentration of trypsin than that to which they were exposed in the trypsinization assay did not significantly affect their subsequent photonegative behavior in the presence of unaltered MH (Table I).

DISCUSSION

Chemical influences on the sign of an organism's phototaxis are not unknown. Thorson (1964) showed that a reduced salinity could reverse the normally positive phototaxis of various pelagic marine larvae. Lucas (1936) demonstrated that the copepod *Eurytemora hirundoides* reversed its positive phototaxis in the presence of abundant diatoms. In the present study adult female *Unionicola formosa* changed

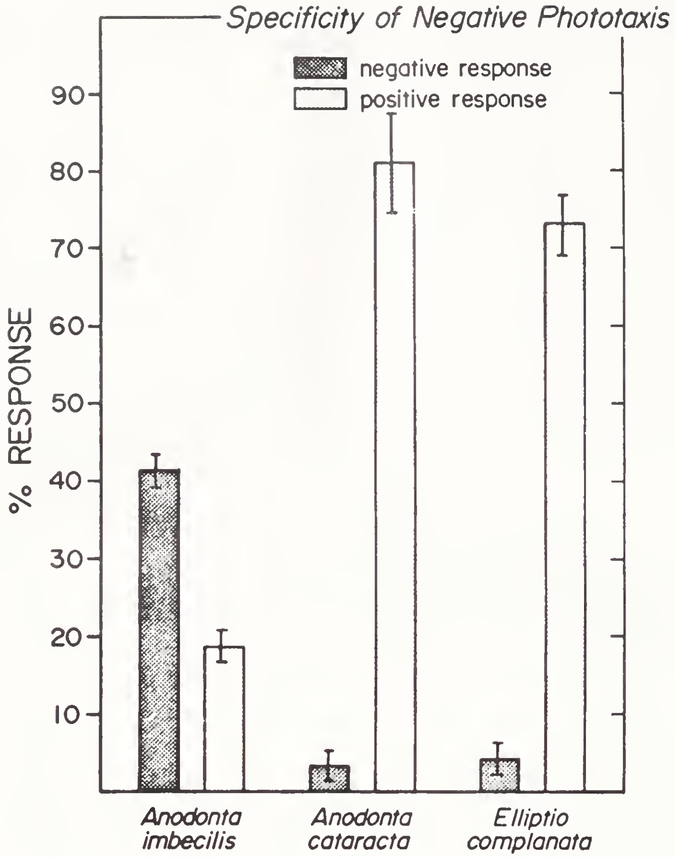

FIGURE 2. Specificity of the negative phototaxis of *Unionicola formosa* induced by mantle homogenate (6.67 μg protein/ml) from various unionid bivalves. Histograms depict $\overline{X} \pm SE$ ($N = 90$). The total for positive and negative response is less than 100% because of the design of the bioassay (see text).

the sign of their phototactic response from positive to negative in the presence of a chemical influence of their host, the freshwater mussel *Anodonta imbecilis.*

The negative phototactic behavior of *U. formosa* followed a more or less classical dose-response relationship with the threshold for negative phototaxis occurring in mantle homogenate with approximately 0.7 μg protein/ml. The response quickly saturated at supra-threshold concentrations (Fig. 1), suggesting that the chemoreceptors involved in the response become saturated at concentrations of the stimulus that are only slightly above threshold. However, there may be a more concentration-dependent aspect of the response, such as some photokinetic phenomenon, that has not been identified.

The host specificity of this induced negative phototaxis was very pronounced. *U. formosa* did not become negatively phototactic either in the presence of water from the mantle cavity or in mantle homogenate (8 times the minimum effective concentration from *A. imbecilis*) of *Elliptio complanata* or of *Anodonta cataracta*, a sympatric congener of the mite's host. These results are only partially in agreement with those of Welsh (1930, 1931) which indicated that *U. formosa* responded only to *A. cataracta.* Whether or not these differences reflect methodological, geographical, or possibly taxonomic considerations is unresolved.

The behavioral specificity that we have observed in this study could potentially be a consequence of one of the following. First, the substance(s) that induces the negative phototaxis could indeed be species-specific and *U. formosa* has evolved sensory capabilities that enable it to respond only to *A. imbecilis.* Secondly, the active substance could be produced by all three species of mussels that were examined but at an ineffective concentration in *A. cataracta* and *E. complanata.* Finally, the three species each could produce the substance to which *U. formosa* responds, but the substance produced by *A. cataracta* and *E. complanata* might be masked by another agent or some inhibitor may prevent the phototactic response by *U. formosa.*

Aside from the preliminary work of Welsh (1930) that suggested that the active substance in preparations of *A. cataracta* was unaffected by boiling and resisted extensive putrefaction at 37.5°C, no information has been available on the chemical nature of any molluscan product that affects phototaxis of unionicolid water mites. We have now shown that the photobehavior of *U. formosa* is modified by some substance from the mantle of *A. imbecilis* that is heat labile and sensitive to trypsinization. The results presented here indicate that the active substance has a molecular weight of >10,000 daltons; additional data suggest that its molecular weight is probably well in excess of 10^5 daltons (del Portillo and Dimock, unpublished observations). These characteristics are consistent with the active substance being proteinaceous. Further analysis of the quantitative and qualitative characteristics of this substance may elucidate how the specificity of this phototactic behavior is mediated.

TABLE I

Effects of exposure to various chemical stimuli on the sign and magnitude of phototaxis by Unionicola formosa. *Entries are the mean ± SE for N = 90. All MH prepared from* Anodonta imbecilis.

Stimulus	Response of *U. formosa* % Positive phototaxis	 % Negative phototaxis
APW	88.8 ± 1.1	0.0
Unaltered MH	20.6 ± 1.8	42.6 ± 3.0
Ultrafiltered MH		
>10^4 daltons	10.0 ± 1.9	64.4 ± 2.2
<10^4 daltons	66.7 ± 1.7	2.2 ± 1.1
Heat-treated MH		
100°C, 30 min	74.7 ± 2.9	9.0 ± 2.9
80°C, 30 min	80.0 ± 3.8	6.7 ± 0.0
60°C, 30 min	30.0 ± 1.9	31.1 ± 1.1
Trypsinized MH		
0.01 μg trypsin/ml, 25°C, 2 h	55.4 ± 4.9	4.6 ± 3.1
Trypsin Controls		
APW + 0.01 μg trypsin/ml	90.7 ± 2.3	0.0
[a]Pre-treated mites + MH	9.0 ± 3.9	39.0 ± 7.4

[a] Mites were incubated for 1 h in 10 μg trypsin/ml then were tested in the presence of MH.

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COLONY SPECIFICITY IN THE ASCIDIAN, *PEROPHORA SAGAMIENSIS*[1]

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ABSTRACT

The presence of colony specificity, *i.e.*, fusion-incompatibility, was revealed in *Perophora sagamiensis.*

When two fusible stolons made a tip-to-side contact, a protrusion appeared from the lateral portion of a stolon. The tests and epidermides of both stolons fused and blood exchange was established. When two nonfusible stolons came into contact, the tip of a stolon became inflated, and the lateral portion of the other stolon protruded with thickened epidermis. After a decrease in the blood stream, cellular parts of both stolons regressed.

Two types of nonfusion were found in *Perophora sagamiensis* and were termed "nonfusion type A" and "nonfusion type B," respectively. In nonfusion type A, two stolons rejected each other without fusion between the tests; while in nonfusion type B, rejection occurred after a transitory fusion of the tests. A given colony showed nonfusion type A to some colonies, and nonfusion type B to some other colonies.

Histological study of the process of nonfusion revealed amoebocytes and lymphocyte-like cells in the contact area.

INTRODUCTION

Multicellular invertebrates, from sponges to protochordates, can recognize self and non-self distinctly (see Hildemann *et al.*, 1979, for review). Furthermore, some of them display allogeneic polymorphism and specific memory in transplantation immunity (Cooper, 1970; Hildemann *et al.*, 1980a, b).

Ascidians, a group of the subphylum Protochordata, respond to foreign bodies in various ways. In the body fluid of some solitary ascidians, many humoral substances have been reported, as represented by a nonspecific precipitin to rabbit serum (Cantacuzene, 1913), agglutinins to foreign spermatozoa (Tyler, 1946), agglutinins to mammalian erythrocytes (Fuke and Sugai, 1972), and natural bactericidins (Johnson and Chapman, 1970). Several types of cellular response have also been described. Small particles of dyes (Ivanova-Kazas, 1966; Smith, 1970; Anderson, 1971) and certain bacteria (Thomas, 1931) are phagocytized. Large particles of dyes (Ivanova-Kazas, 1966) and inserted glass fragments (Anderson, 1971) are encapsulated by blood cells. In some solitary ascidians allograft rejection is accompanied by the infiltration of vanadocytes (Anderson, 1971) or by that of lymphocyte-like cells at the interface (Reddy *et al.*,, 1975).

Allogeneic recognition in colonial ascidians has been well documented by the study of colony specificity, manifested by fusion-incompatibility between two col-

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onies. Some colonies fuse to form a single mass, but others do not. In *Botryllus primigenus*, the fusibility was shown to be under genetic control (Oka and Watanabe, 1957, 1960, 1967). In all compound ascidians studied so far, contact between fusible colonies results in the union of both the tests and the common vascular systems (if present) of the two colonies. However, there are some differences among species in the mode of rejection. In *Botrylloides simodensis* (Mukai and Watanabe, 1974) and *Perophora japonica* (Koyama and Watanabe, 1981), the tests never fuse between nonfusible colonies. In *Botryllus primigenus* (Oka and Watanabe, 1957) and *Didemnum moseleyi* (Mukai and Watanabe, 1974), rejection always occurs after fusion between the tests of two colonies; whereas in *Botryllus scalaris* rejection occurs after a fusion not only between the tests but also between the test vessels (Saito and Watanabe, 1979). (For the scientific names of *Botryllus scalaris* and *Botrylloides simodensis*, see Saito *et al.*, 1981a, b.)

We have been studying colony specificity in some Japanese species of the Perophoridae. In *Perophora formosana* colony specificity is absent (Mukai and Watanabe, 1974); while colony specificity is present and is expressed in the test surface in *Perophora japonica* (Koyama and Watanabe, 1981). In the present study, we describe the processes of fusion and nonfusion observed in *Perophora sagamiensis.*

MATERIALS AND METHODS

Several living colonies of *Perophora sagamiensis* were collected from Hatakejima Island in Tanabe Bay, Wakayama, Japan. They were attached to microscopic slides and reared in a bay near the Shimoda Marine Research Center, Shizuoka, Japan. In a colony, respective zooids are connected with one another by vascular stolons from which new individuals arise as small buds. The stolons are sometimes branched and interconnected within a colony, giving a mesh-like appearance.

Experimental procedures were similar to those described in a previous paper (Koyama and Watanabe, 1981); two zooid-stolon systems were tied upon a glass slide, about 1 cm apart and at about right angles to each other, so as to make a contact between the tip of one stolon and the lateral portion of the other stolon. In this paper, the former stolon will be termed "t-stolon" and the latter stolon "l-stolon." Repeated observations were made following the onset of a tip-to-side contact between the two stolons.

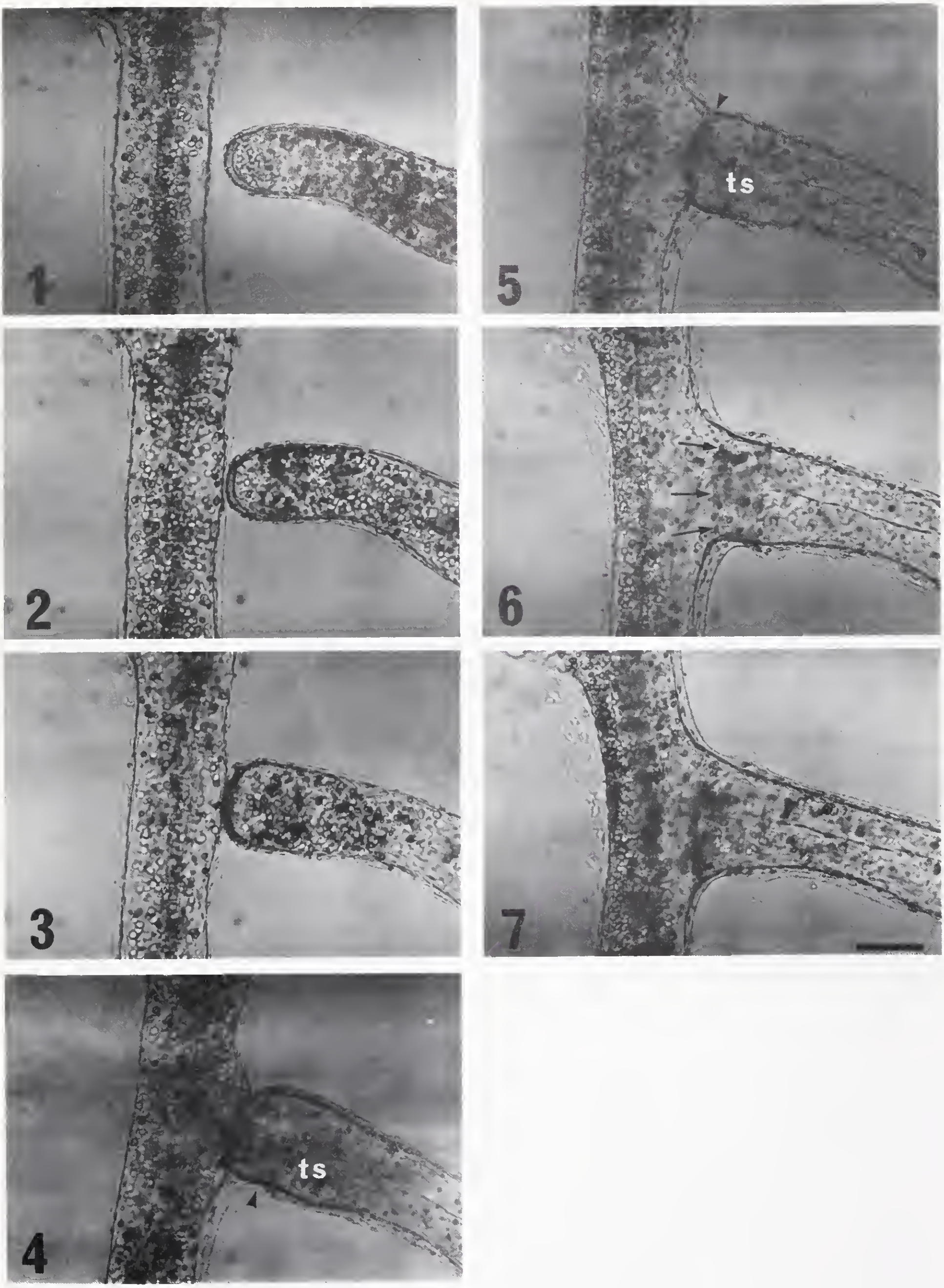
For the histological study, some specimens in the process of nonfusion were fixed in Bouin's solution made in sea water, dehydrated through a butanol series and embedded in paraffin. They were sectioned at 4 μm and stained with Delafield's haematoxylin and eosin-orange G.

RESULTS

The process of fusion

When a tip-to-side contact was made between two stolons derived from a colony, fusion always took place. A typical example of fusion is shown in Figures 1–7.

No change was observed in either stolon prior to contact (Fig. 1). After contact the tip became somewhat flat (Fig. 2). Then, a protrusion appeared in the lateral portion of the l-stolon (Fig. 3). Five hours after contact (Fig. 4), in the t-stolon the tip became inflated and the terminus of the septum (ts) was near the contact area. The protrusion of the lateral portion increased in height, and the test of the l-stolon encroached upon the test of the t-stolon (arrowhead). Six hours after contact



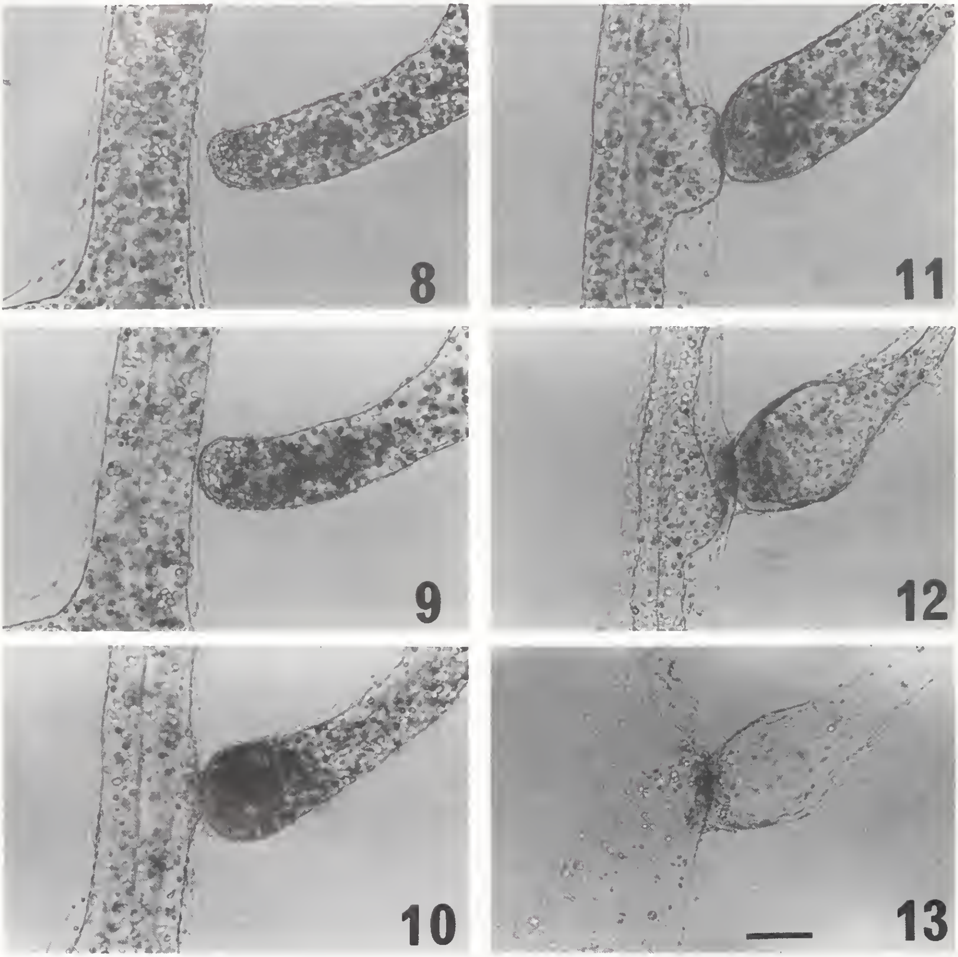
FIGURES 1–7. A typical example of fusion. (Fig. 1): 50 min before contact. (Fig. 2): 16 min after contact. (Fig. 3): 2 h after contact. (Fig. 4): 5 h after contact. The arrowhead indicates the test of the l-stolon encroached upon the test of the t-stolon. ts, terminus of the septum in the t-stolon. (Fig. 5): 6 h after contact. The arrowhead shows the union of epidermis between the two stolons. ts, terminus of the septum in the t-stolon. (Fig. 6): 8 h after contact. The arrows indicates epidermal cells remaining in the anastomosing area. (Fig. 7): 10 h after contact.

All figures are the same magnification; the bar in Figure 7 is 100 μm long.

(Fig. 5), a small amount of blood interchange was established between the two stolons. The epidermides of both stolons were partly connected (arrowhead). In the t-stolon, the terminus of the septum (ts) was almost at the contact area. As fusion proceeded both between the tests and between the epidermides, the remaining epidermal cells (arrows) in the anastomosing area were gradually eliminated (Fig. 6). Then, fusion was completed both in the test and in the epidermis, and the remaining epidermal cells disappeared (Fig. 7).

The process of nonfusion

In *Perophora sagamiensis*, we found two types of nonfusion. One will be termed "nonfusion type A" and the other "nonfusion type B" hereafter. In nonfusion type A, the two stolons reject each other without fusion between their tests; while in nonfusion type B, rejection occurs after a transitory fusion of the tests.



FIGURES 8–13. A typical example of nonfusion type A. (Fig. 8): 45 min before contact. (Fig. 9): Immediately after contact. (Fig. 10): 8 h after contact. (Fig. 11): 24 h after contact. (Fig. 12): 96 h after contact. (Fig. 13): 136 h after contact.

All figures are the same magnification; the bar in Figure 13 is 100 μm long.

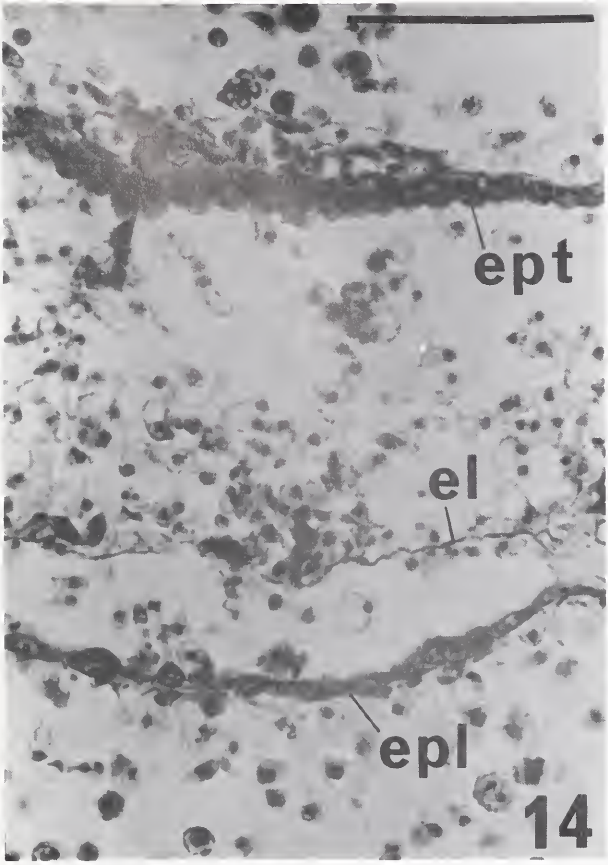
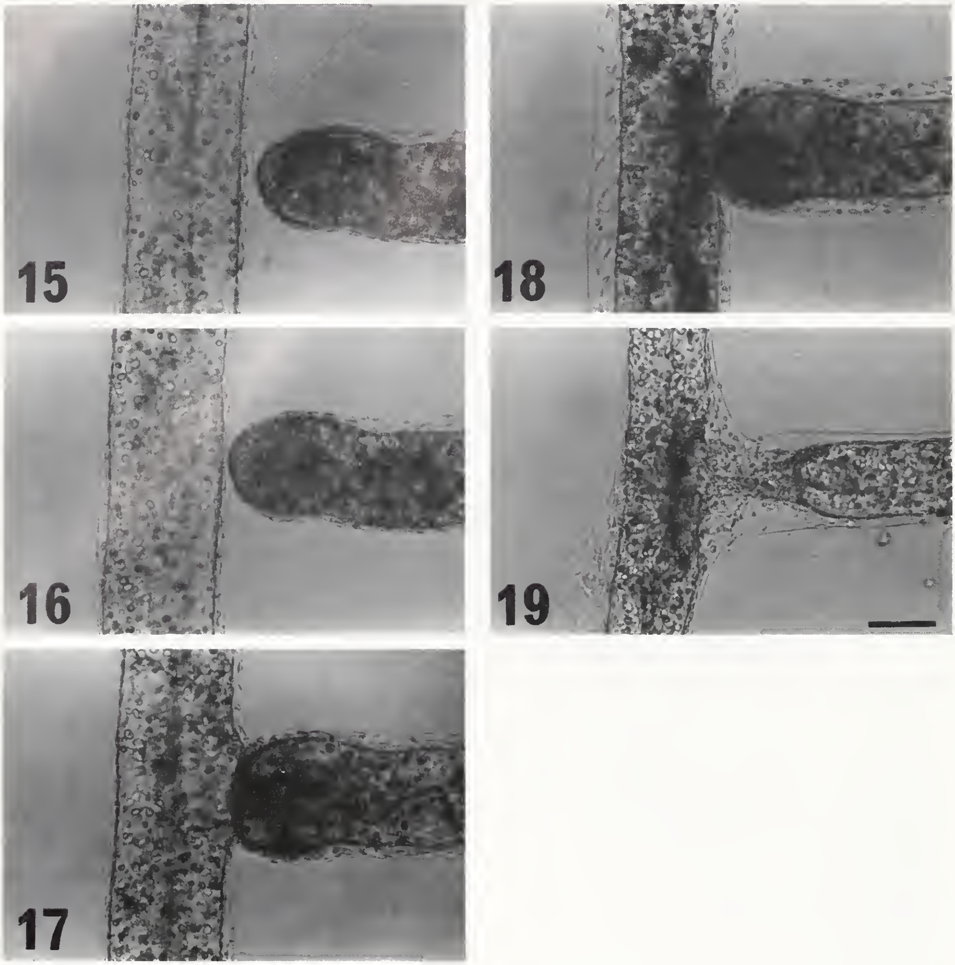


FIGURE 14. A histological figure of nonfusion type A, after detachment of the two stolons. el, external layers of the test; epl, thickened epidermal cells of the l-stolon; ept, epidermal cells of the t-stolon. Scale bar = 50 μm.

Figures 8–13 show a typical example of nonfusion type A. The t-stolon was growing to the l-stolon without a noticeable change (Fig. 8). Then, a contact was made between the two stolons (Fig. 9). After contact, a protrusion appeared in the lateral portion (Fig. 10). The epidermis of the protrusion thickened. Twenty-four hours after contact (Fig. 11), the dilatation of the tip and the protrusion of the lateral portion were at maximum, but attenuation of the stolon had already begun. Later, distal parts of both stolons became thinner and their blood stream decreased (Fig. 12). After that, the cellular parts of both stolons regressed (Fig. 13), leaving empty test tubes in the original place. Disintegration of interacting stolons was not observed, unlike in the case of nonfusion of *Botryllus primigenus* (Oka and Watanabe, 1957).

Throughout the reaction of nonfusion type A described above, a clear demarcation line was obvious between the two stolons. Furthermore, at any stage of the reaction, the two stolons could readily be separated from each other by pulling them without causing noticeable damage. Even after two stolons were detached, the external layers of the test could be recognized (Fig. 14). From these observations, it may be concluded that no union of test matrices is established in nonfusion type A.



FIGURES 15–19. Serial photomicrographs of nonfusion type B. (Fig. 15): 15 min before contact. (Fig. 16): Immediately after contact. (Fig. 17): 3 h after contact. (Fig. 18): 4 h after contact. (Fig. 19): 28 h after contact.

All figures are the same magnification; the bar in Figure 19 is 100 μm long.

Figures 15–19 are serial photomicrographs of nonfusion type B. When the t-stolon was growing toward the l-stolon, no specific change was observed in either of them (Fig. 15). Soon, the two stolons came into contact (Fig. 16). Then, a protrusion appeared in the lateral portion (Fig. 17). After a while, the two stolons dwindled and their blood stream decreased (Fig. 18). Finally, the cellular parts of the two stolons detached from each other (Fig. 19) and regressed.

In the later stages of nonfusion type B, the demarcation line between the two stolons became obscure and the test tubes of the two stolons were no longer separable. Histologically, no external layers of the test could be recognized between the epidermal cells of the two stolons (Fig. 20). Moreover, in the case shown in Figure 20, there was a bridge of epidermal cells connecting the two stolons. These results may be accepted as evidence for the fusion of the tests in nonfusion type B.

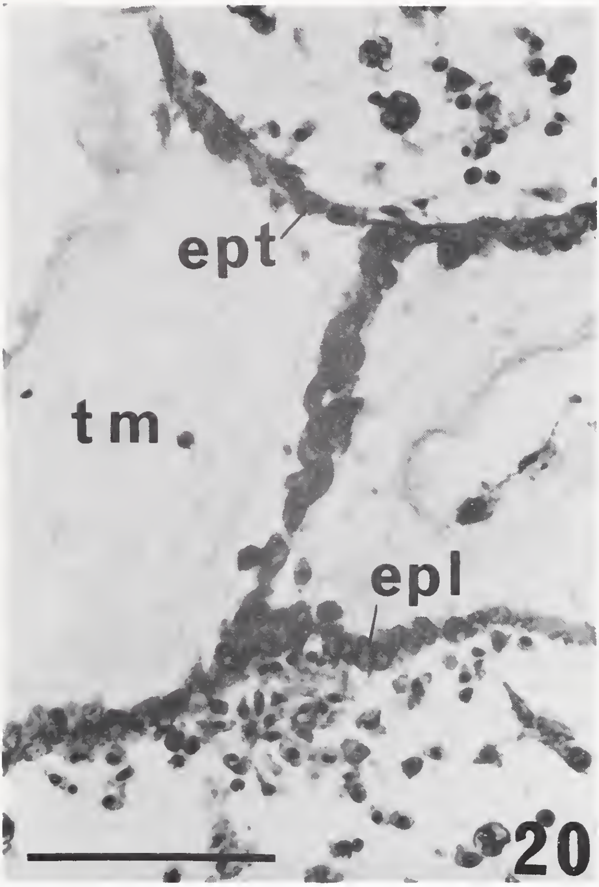


FIGURE 20. A histological figure of nonfusion type B, after detachment of the two stolons. Many amoebocytes were observed in the lumen of the l-stolon. epl, epidermal cells of the l-stolon; ept, epidermal cells of the t-stolon; tm, test matrix. Scale bar = 50 μm.

Fusibility between different colonies

The two types of nonfusion did not take place at random. A particular combination of incompatible colonies always exhibited only one type of nonfusion.

We first tested the fusibility of a certain colony to twelve other colonies. Of the twelve combinations, four produced fusions, five produced nonfusions of type A, and the remaining three produced nonfusions of type B. Next, six colonies were selected and fusibility among them was tested in all possible binary combinations. The results are shown in Table I. For example, Col. 1 fused with Cols. 2, 3 and 4. However, in the combinations among Cols. 2, 3 and 4, only Col. 2 and Col. 4 fused and the other two combinations showed nonfusion type B. When Col. 5 was combined with Col. 1, 2, 3 or 4, nonfusion type B occurred. In every combination with Col. 6, nonfusion type A took place.

Histological observations of nonfusion

Histologically, in either type of nonfusion, two kinds of blood cell were often observed near or on the epidermal cells of the contact area. They were amoebocytes

TABLE I

The fusibility between different colonies taken from nature.

	Col. 1	Col. 2	Col. 3	Col. 4	Col. 5	Col. 6
Col. 1	f	f	f	f	nf B	nf A
Col. 2	f	f	nf B	f	nf B	nf A
Col. 3	f	nf B	f	nf B	nf B	nf A
Col. 4	f	f	nf B	f	nf B	nf A
Col. 5	nf B	nf B	nf B	nf B	f	nf A
Col. 6	nf A	nf A	nf A	nf A	nf A	f

f = fusion, nf A = nonfusion type A, nf B = nonfusion type B.

and lymphocyte-like cells (Figs. 14, 20, and 21). They were observed from four hours after contact and even after detachment of the stolons. The amoebocytes had a distinct nucleus and clear cytoplasm. They were spherical, flattened, or irregular in shape. The nearly spherical lymphocyte-like cells, being 3–4 μm in diameter, had a prominent nucleus and a small amount of basophilic cytoplasm. Their nuclei were round or pear-shaped with a few clusters of chromatin.

DISCUSSION

These results demonstrated that colony specificity is present in *Perophora sagamiensis.*

The processes of fusion and nonfusion type A in this species are similar to those of fusion and nonfusion, respectively, observed in *Perophora japonica* (Koyama

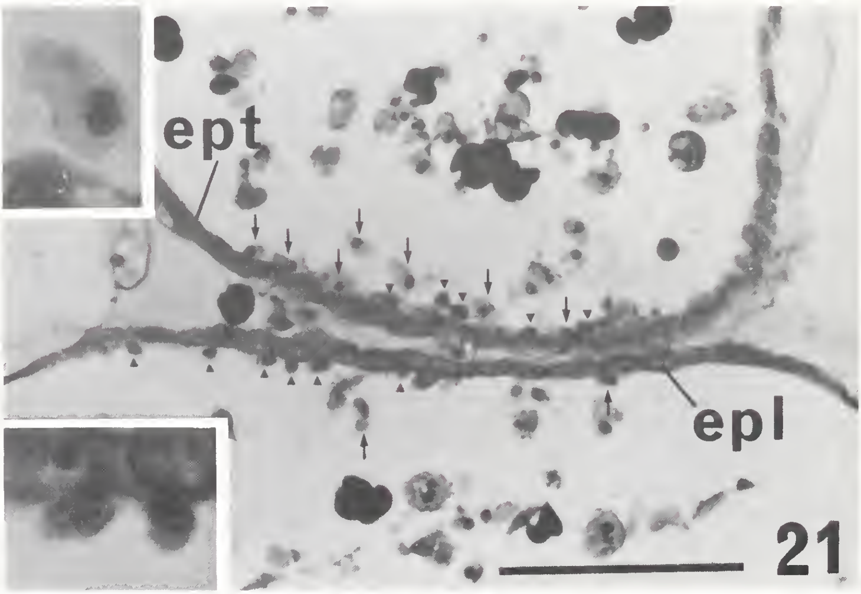


FIGURE 21. Amoebocytes (arrows) and lymphocyte-like cells (arrowheads) near and on the epidermal cells in the contact area in nonfusion type B. 4 h after contact. epl, thickened epidermal cells of the l-stolon; ept, epidermal cells of the t-stolon. Scale bar = 50 μm. Upper inset, a magnified amoebocyte. Lower inset, two magnified lymphocyte-like cells on the epidermal cells.

and Watanabe, 1981). From the present and previous works (Mukai and Watanabe, 1974; Koyama and Watanabe, 1981), the three species of Japanese Perophoridae can be divided into two groups: one without colony specificity and the other having it. *Perophora formosana* belongs to the former, and *P. japonica* and *P. sagamiensis* to the latter group. From the observations of the zooid structure, the way of reproduction and the morphology of blood cells, *Perophora formosana* can be clearly distinguished from the other two species (Tokioka, 1953; and our unpublished data).

The results of the present and the previous fusion experiments in several colonial ascidians are summarized in Table II. In the reaction resulting from contact between natural surfaces of two colonies in respective species, three cases can be distinguished. One case is fusion, in which complete fusion both in the test and in the test vessels is established between the two colonies; another case is rejection, in which some physiological antagonism is seen between the two colonies; the last case in which no particular reaction can be observed is referred to as indifference. Colonial ascidians so far studied can be classified into two major groups. In one group, to which *Polycitor mutabilis* and *Perophora formosana* belong, colony specificity is absent: when two colonies come into contact, indifference occurs between natural surfaces, whereas fusion takes place between cut surfaces, regardless of their origin. In the other group colony specificity is present. Discrimination of self and non-self is made between two colonies, resulting in fusion, rejection, or indifference. This group consists of several subgroups. In the first subgroup, discrimination occurs before fusion between the tests of two colonies. *Botrylloides simodensis* and *Perophora japonica* belong to this subgroup. In the second subgroup, to which *Botryllus primigenus* and *Didemnum moseleyi* belong, discrimination

TABLE II

Summarizing representation of fusion experiments. The "discrimination points" in the text are shown by braces.

Species	Test	Test (blood) vessel	Colony	Authors
Polycitor mutabilis	Nonfusion		Indifference	Oka and Usui, 1944
Perophora formosana	Nonfusion		Indifference	Mukai and Watanabe, 1974
Botrylloides simodensis	Fusion	Fusion	Fusion	Mukai and Watanabe, 1974
	Nonfusion		Indifference	
Perophora japonica	Fusion	Fusion	Fusion	Koyama and Watanabe, 1981
	Nonfusion		Rejection	
Perophora sagamiensis	Fusion	Fusion	Fusion	
		Nonfusion	Rejection (Type B)	
	Nonfusion		Rejection (Type A)	
Botryllus primigenus	Fusion	Fusion	Fusion	Oka and Watanabe, 1957
		Nonfusion	Rejection	
Didemnum moseleyi	Fusion		Fusion	Mukai and Watanabe, 1974
			Rejection	
Botryllus scalaris	Fusion	Fusion	Fusion	Saito and Watanabe, 1979
			Rejection	

takes place after fusion between the tests but before fusion between the test vessels (if present). In the third subgroup discrimination occurs after fusion between the test vessels of two colonies. *Botryllus scalaris* belongs to this subgroup. In all species mentioned above, there is a single discrimination point. In *Perophora sagamiensis*, there are two discrimination points, *i.e.*, before and after fusion between the tests. Therefore, this species has two features, one shared with the first subgroup and the other with the second subgroup.

As seen in Table I, a given colony will show nonfusion type A to some colonies and show nonfusion type B to some other colonies. This may suggest that the fusibility of the test and that of the blood vessel are controlled separately. Type B nonfusible colonies seem to share only the fusibility of the test, and type A nonfusible colonies do not. Detailed genetic analysis is desirable to clarify the controlling factors of fusibility in this species.

Histological study of the process of nonfusion revealed the presence of amoebocytes and lymphocyte-like cells near or on the epidermal cells in the contact area. These two types of blood cell were observed to behave likewise during the process of fusion (unpublished data). It is tempting to speculate that they might participate in the recognition of self and non-self. In *Ciona intestinalis*, infiltration of lymphocyte-like cells accompanied by allograft rejection was observed and their roles in allogeneic recognition and rejection have been discussed (Reddy *et al.*, 1975). Much more study is needed, however, to elucidate the significance of these blood cells in the processes of fusion and nonfusion.

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THE CELLULAR STRUCTURE OF THE LEYDIG ORGAN IN THE SHARK, *ETMOPTERUS SPINAX* (L.)

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Abstract

In *Etmopterus spinax*, a small deep water shark, the predominating lymphomyeloid tissue is the so called Leydig organ. This consists of bone marrow-like tissue situated between the muscularis and the mucosa of the esophagus. Examination by light and electron microscopy shows that the Leydig organ produces large numbers of granulocytes and lymphocytes. Two main types of granulocytes occur, tentatively called heterophilic and eosinophilic. The heterophilic cells may be subdivided into three types which differ in the ultrastructure of the granules. Cells structurally resembling mammalian plasma cells are common. The presence of these cells indicates that the tissue is part of the shark immune system.

Introduction

In 1685 the Danish anatomist, Nicolaus Steno, noticed a gland-like structure in the esophagus of a ray, *Raja*. Probably the same structure was later found in several elasmobranchs, and Leydig (1857) interpreted it as a lymph node. Later authors ascribe it both lymphoid and myeloid activities. Therefore the term lymphomyeloid may be appropriate to describe this kind of tissue. The esophageal lymphomyeloid tissue of elasmobranchs is often called the Leydig organ (organ of Leydig). In some elasmobranchs the Leydig organ is remarkably large. Bolton (1927) found it to weigh about 1.6 kg in one 1.8 m long cow shark (*Hexanchus corinus*), and 1.2 kg was noted in a 2.9 m Greenland shark (*Somniosus microcephalus*) by one of the authors (R.F.).

Certain elasmobranchs lack the Leydig organ but instead possess lymphomyeloid tissue associated with the gonads, the epigonal organ. Most species of elasmobranchs possess both Leydig and epigonal organs.

In recent years a growing number of investigators have become interested in the evolution of the immune system. It has been clear that elasmobranchs show strong immune responses (see review by Marcharlonis, 1977). In connection with the findings of immunological activity it is important to reinvestigate the various lymphomyeloid structures which form the structural basis of immune processes.

Histological studies of the Leydig organ have been done by Pilliet (1890), Drzewina (1905, 1910), Petersen (1908), Kulchitzkii (1911), Maximov (1923), Kanesada (1956), Fey (1965) and Fänge (1968). The ultrastructure of the Leydig organs of the rays *Raja clavata* and *Torpedo marmorata* has been investigated by Zapata (1981) and of the dogfish, *Scyliorhinus canicula* (by R.F. and A.P., Marine Biol. Assoc. UK, 1981, in preparation).

The shark, *Etmopterus spinax*, belongs to those species of elasmobranchs which have a well developed Leydig organ but lack the epigonal lymphomyeloid structure. The present study describes the ultrastructure of cells from the Leydig organ of

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Etmopterus spinax. In addition we discuss histological data obtained from a material larger than that used earlier (Fänge, 1968). By using both light microscopical and ultrastructural criteria most cells can be equalized with those from mammalian lymphomyeloid systems.

MATERIALS AND METHODS

The animal material consisted of about 50 specimens of the blue velvet, *Etmopterus spinax*, a small deep water shark occurring in the North Atlantic. The body weights varied between 12 g to 600 g (adult females). The fishes were caught in the Scagerack Sea from depth of 200–400 m by trawling. (A few specimens were infested by the cirripedian parasite, *Anelasma squalicola*). Fresh samples of tissues were prefixed on board the ship.

For histology pieces of tissues were fixed in 10 per cent neutral formalin and examined by routine methods after staining with eosin-haematoxylin or Giemsa. Imprints (touch preparations) were air dried 1 hour, fixed for 15 min in absolute methanol and stained with May-Grünwald-Giemsa (MGG).

For electron microscopy small cubes of tissue, about 1 mm^3, were fixed in 3 per cent glutaraldehyde, pH 7.3 for 1–3 days. This solution was made up either of 0.2 *M* cacodylate buffer or in a mixture of one third veronal acetate buffer and two thirds sea water. To this latter solution sucrose was added to a final concentration of 5 per cent as was a trace of $CaCl_2$ (*cf.* Bell *et al.*, 1969). All samples were postfixed in 1 per cent OsO_4 dissolved in sea water. After dehydration in a series of ethanol the cubes were embedded in Epon 812 and sectioned on an LKB ultrotome. The sections were contrasted with uranyl acetate followed by lead citrate and examined in a Hitachi HS-8 electron microscope at magnifications varying from 1,900× to 47,000×. The study is based on more than 500 electron micrographs. Thick sections (about 1 μm) adjacent to the thin sections studied in EM were stained with toluidine blue for light microscopy.

RESULTS

Light microscopy

The Leydig organ constitutes two whitish masses in the dorsal and ventral parts of the esophagus (Fig. 1). The tissue consists of enormous numbers of mature and immature leucocytes within a connective tissue stroma. Lobes formed by tightly packed cells are separated by irregular venous spaces. The lobes are penetrated by small arteries. Histologically the tissue resembles hemopoietic bone marrow of terrestrial vertebrates, although it does not form red cells and contains no fat tissue. As previously found (Fänge, 1968) the predominating cells are two types of granulocytes and non-granulated cells with basophilic cytoplasm. The heterophilic granulocytes are more frequent than the eosinophilic ones. The heterophilic granulocytes contain fine rod-shaped weakly eosinophilic granules. In imprints these cells measure 17–25 μm (width) by 20–28 μm (length), whereas the same cells appear about half that size in histological sections (9–12 $\mu m \times 10$–15 μm). The eosinophilic granulocytes are of similar size but usually more round. They contain rather large (1–2 μm) intensely eosinophilic granules (Fig. 2A). The majority of the granulocytes appear to be in the myelocyte stage and often show mitoses.

Types of non-granulated cells identified in MGG-stained imprints are lymphocytes of various sizes, spindle-shaped cells (thrombocytes), blast cells (probably mainly granuloblasts), plasma cells and monocytes. However, the separation by

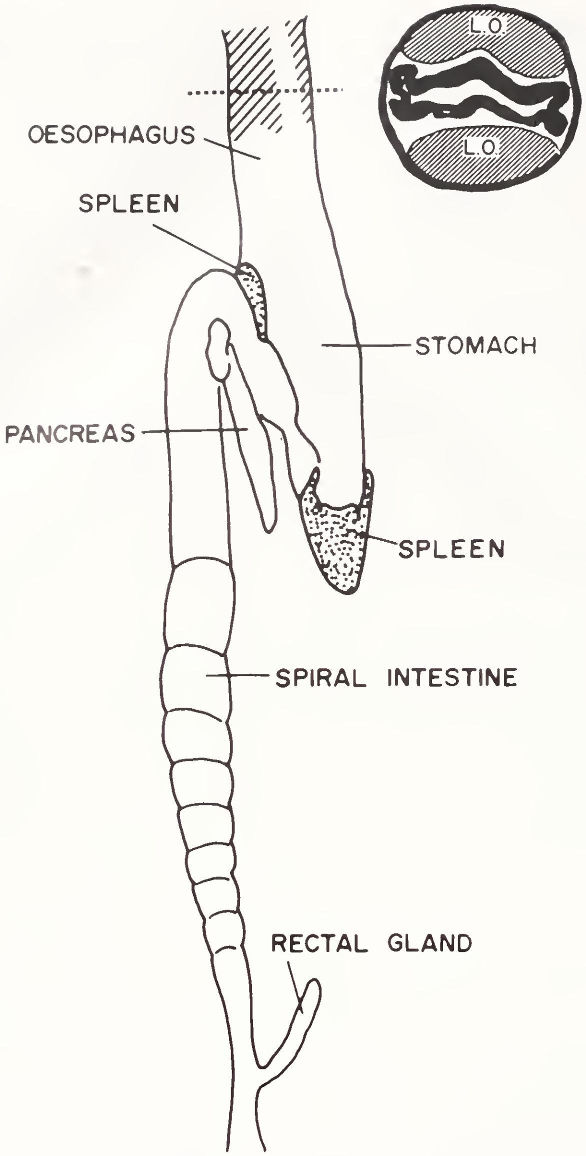

FIGURE 1. Ventral view of the digestive tract showing the localization of the Leydig organ (shaded). To the right an enlarged and transversal section through the esophagus (at the broken line) showing dorsal and ventral portions of the Leydig organ (L.O.).

light microscopy of blast-type cells and plasma cells is uncertain, since both cell types possess a large nucleus and an intensely basophilic cytoplasm. An eccentric position of the nucleus and a clear zone next to the nucleus are features which may be characteristic of plasma cells. It is also difficult to distinguish between small lymphocytes and spindle cells (thrombocytes).

The leucocytes of the Leydig organ often aggregate into diffuse groups or follicles (Fig. 2B). This especially concerns blast cells, eosinophilic granulocytes and

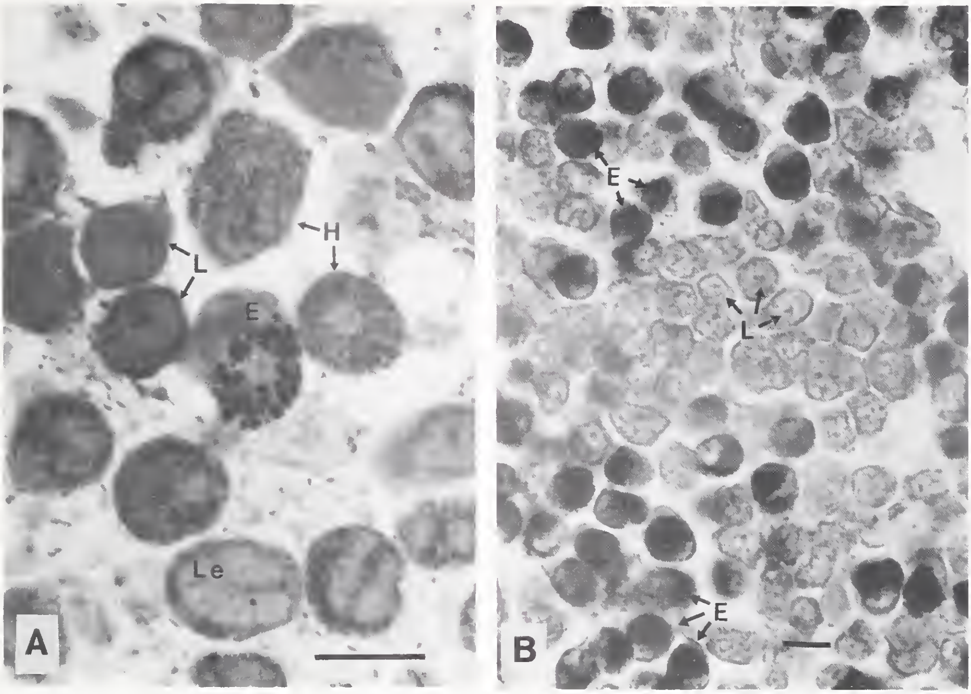

FIGURE 2. Light microscopy of the Leydig organ of *Etmopterus.*

(A): Epon-embedded section, about 1 μm thick and stained with toluidine blue. In the middle an eosinophilic granulocyte (E). Lymphocytes (L) and heterophilic granulocytes (H) are seen around the eosinophilic cell. At the bottom a typical leptomeric cell (Le) with invaginated nucleus. Oil immersion. Bar = 10 μm.

(B): Azan-stained paraffin section. In the middle of the figure a group of non-granulated cells, mainly lymphocytes. Above and below these cells there are eosinophilic granulocytes (dark cells). Bar = 10 μm.

small lymphocytes. In Giemsa-stained histological sections the tissue appears as a mosaic of red-stained granulocytes and bluish non-granulated cells.

Electron microscopy

Most cells of the Leydig organ show ultrastructural characteristics resembling those of blood cells from various vertebrates and even invertebrates (Bessis, 1973; Mattisson and Fänge, 1977). The same types of leucocytes as identified by light microscopy are also found by the electron microscope, but the more structural details obtained are important for a further identification of the leucocytes.

Eosinophilic granulocytes. On electron micrographs the granules measure up to 1.5 μm in diameter and have a spheric form (Fig. 4). In contrast to generally described eosinophils of higher vertebrates those of the *Etmopterus* Leydig organ contain granules without a crystalline core. The periphery of the granules often has an electron lucent frame. Compared with the heterophilic cells described below the eosinophils are less densely packed with granules. Surrounding the granules a rough endoplasmic reticulum is often found (Fig. 4). The eosinophils seem to make up only about 5% of the total number of granulocytes.

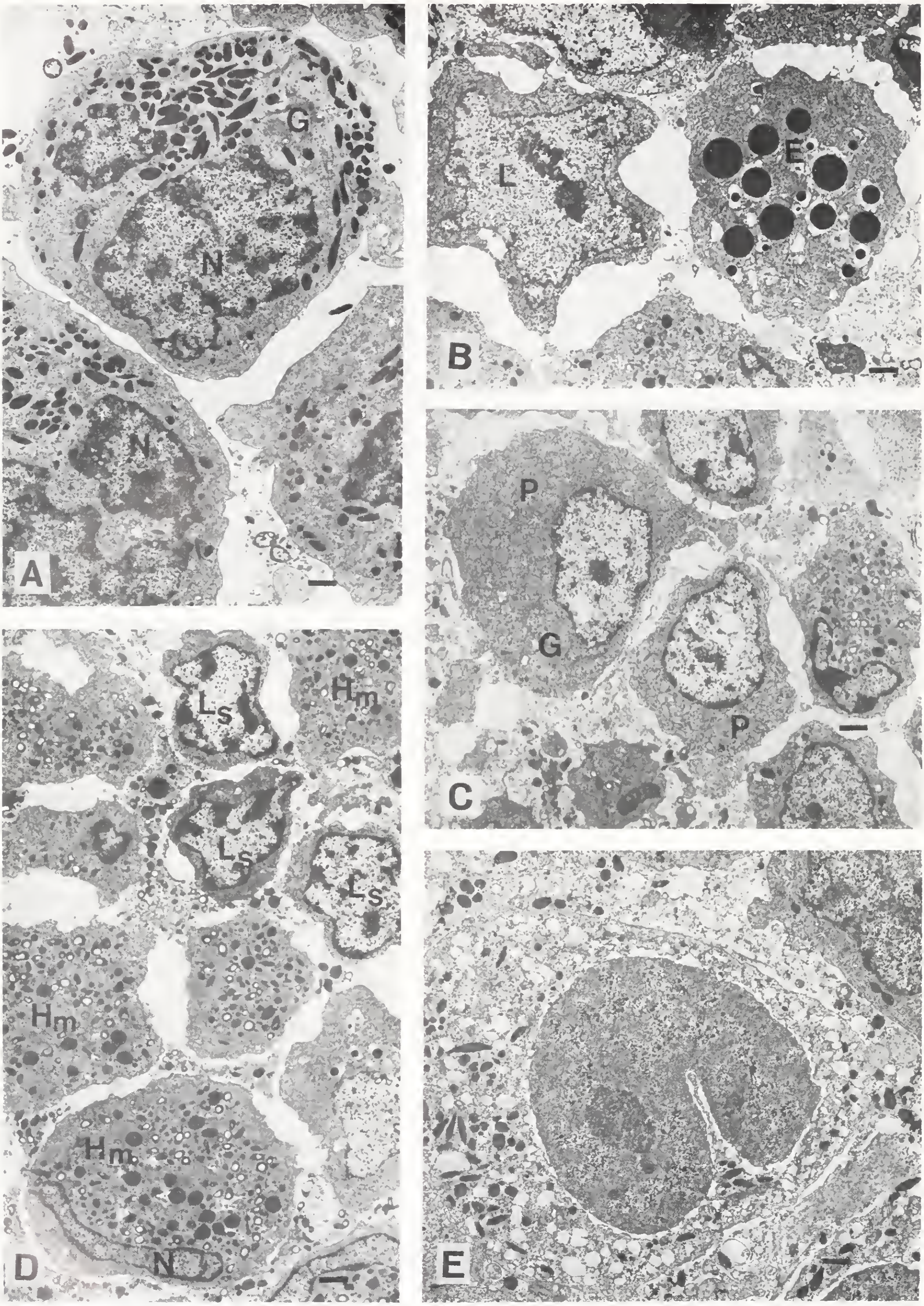

FIGURE 3. Electron microscopy of different cell types from the Leydig organ. Bar = 1 μm.

(A): The most common heterophilic granulocytes, designated type A. Ovoid and rod-shaped granules, a few of them in the intercellular space. Lobed nuclei (N). Golgi apparatus (G). Fixation for 1–3 days in 3% glutaraldehyde dissolved in 0.2 *M* Na-cacodylate buffer. Postfixation for 1 h in 1% OsO_4 in sea water. The fixation media were adjusted to neutral pH.

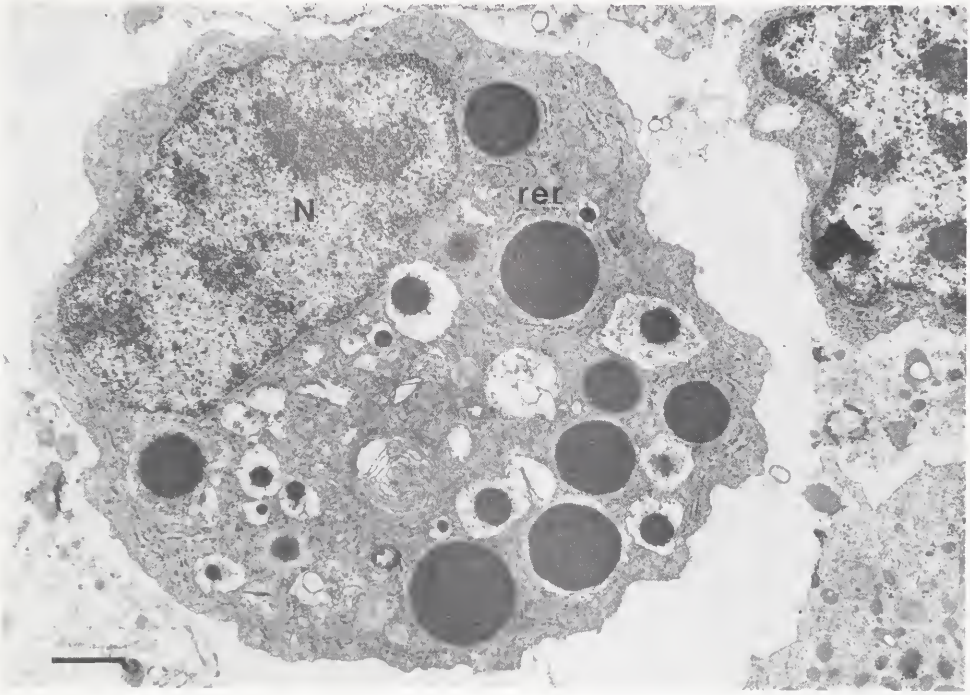

FIGURE 4. Eosinophilic granulocyte. Nucleus (N) at the periphery of the cell. The cytoplasm contains a few spheric and homogeneously dense granules. Each of the smaller dense spheres is surrounded by a "clear area." Between the granules is a well developed rough endoplasmic reticulum (rer). Fixation as in Figure 2B. Bar = 1 μm.

Heterophilic granulocytes. Ultrastructurally the granules are ovoid about 0.5 μm by 1 μm (Figs. 3 and 5). Solitary granules, however, may be rod-shaped with a length 4–6 times their width. As seen in Figure 5 the granules contain filamentous longitudinal internal structures. The heterophils often have a highly lobed nucleus and close to this there are often annulate lamellae. Next to the nucleus there is a well developed Golgi apparatus and numerous vesicular structures, which probably are primary lysosomes. However, the heterophilic granulocytes vary much in

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(B): Large lymphocyte or blast cell (L) and eosinophilic granulocyte (E). The latter has spheric and homogeneously dense granules, some of them surrounded by an "empty" region. Fixation as in Figure 3A but glutaraldehyde was dissolved in a mixture of veronal acetate and sea water (1:2). This solution was supplied with sucrose to a final concentration of 5% and a trace of $CaCl_2$. Adjustment to neutral pH with NaOH.

(C): Two plasma cells (P) dominate the figure. Besides the typical ribosome-coated endoplasmic reticulum the cytoplasm of the left cell has a few dense granules close to the Golgi apparatus. Both cells show "empty" areas within the nuclei. Fixation as in Figure 2B.

(D): Small lymphocytes (L_s) and modified heterophilic granulocytes (H_m). The lymphocyte nuclei have areas with strongly electron dense chromatin. The modified granulocytes, designated type B, show characteristic slender "saucer-shaped" nuclei (N) and granules with central electron lucent regions. Fixation as in Figure 2B.

(E): Another type of modified heterophilic granulocyte, designated C. This type is characterized by a great number of electron lucent areas (degranulated areas?) mingled with the common type of heterophilic granule and it has a leptochromatic nucleus pierced by a string of cytoplasm. Fixation as in Figure 2A.

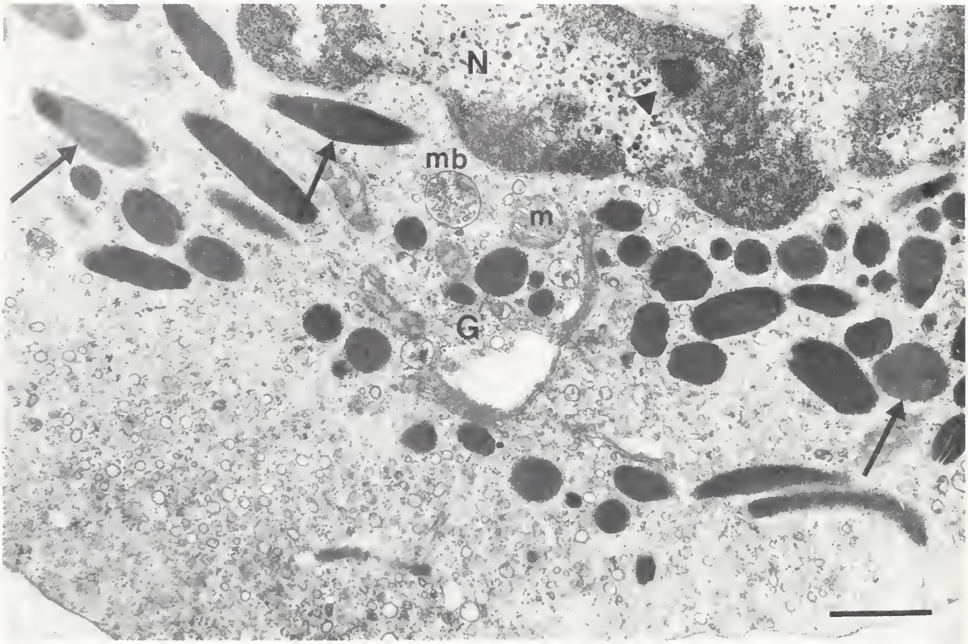

FIGURE 5. Portion of a heterophilic granulocyte (type A). The cytoplasm is well supplied with electron lucent small vesicles and granules of the common heterophilic type, some of them with a filamentous interior (arrows). Nucleus (N) with a granule-like body (arrow head), mitochondria (m), multivesicular body (mb) and Golgi apparatus (G). Fixation as in Figure 2A. Bar = 1 μm.

their ultrastructure. Based on differences in the structure of the granules we tentatively distinguish three sub-types of heterophilic granulocytes, here designated types A, B and C.

Type A. (Figs. 3A, 5). The granules of this type of heterophilic cell are homogeneously electron dense. Type A cells constitute about two thirds of the total number of heterophils. However, different types of heterophils occupy special areas within the Leydig organ and therefore this estimation of the number of type A cells is only an approximation.

Type B. (Fig. 3D). The cells are smaller than those of A-type. The smaller granules have a central vesicular structure with electron lucent interior. The B-cells have a very characteristic saucer-shaped nucleus.

Type C. (Fig. 3E). These cells are 6–10 μm in diameter, have a very loose cytoplasm and show degenerate features. The cytoplasm contains membrane-bound vesicles with a size and shape agreeing with the granules of the other heterophils. Some of the cells show a highly reduced electron density all over the cells. Type B- and type C-cells are found in about the same number. In both B- and C-cells there often occur granules of the common heterophilic type (type A-cells).

With electron microscopy we often found free intact granules in interstitial regions (Fig. 3A). The presence of free leucocytic granules is also noticed in almost all imprint preparations and has previously been reported by Fey (1966) from the dogfish (*Scyliorhinus canicula*). Besides these free granules a special type of spheric body occurs between the cells as well as inside cells of monocyte type. These granules may be phagocytic inclusions or they may be formed inside the cells. They are up to 2 μm in diameter and have a moderate density suggestive of lipids or lipoproteins (Fig. 6). When occurring in the cytoplasm the bodies are surrounded by a circle of dense irregular grains.

Small lymphocytes in the electron microscope measure 4–6 μm in diameter and have a large nucleo-cytoplasmic ratio (Fig. 3D). The nucleus has a dense, mainly peripheral heterochromatin and the cytoplasm has a high content of free ribosomes. Another category of lymphocyte-like cells measures 8–12 μm in diameter. Their more extensive cytoplasm is richly supplied with free ribosomes as well as with polysomes. The nucleus has a well developed nucleolus. These cells may be large lymphocytes or blast cells. As granulocytes are predominant, many blast cells probably are granuloblasts (Fig. 3B).

Among the lymphocyte-like cells there are some which are difficult to identify with known cell types. Some of them have granules similar to those of heterophilic granulocytes. Others have a leptochromatic nucleus pierced by a cytoplasmic invagination. The latter may be "pale transition cells" (Yoffey, 1980).

Cells containing vacuoles of varying sizes, microtubules, filaments and a special type of granules may be considered as monocytes (Fig. 6). Their specific granules are about 0.2 μm in diameter and supplied with a thin halo. This type of granule is often, *e.g.* by Bessis (1973), described as azurophilic granules. In addition to the azurophilic granules one also observes granules with lipid density.

The plasma cells, or plasma cell-like cells, measure up to 15 μm in diameter. The cytoplasm shows an ultrastructure similar to that of mammalian plasma cells (Figs. 3C, 7). A well developed Golgi apparatus and a vast system of rough endoplasmic reticulum reflects a high level of protein synthesis. The reticulum forms sac-like structures richly supplied with ribosomes (Inset Fig. 7). Among the endoplasmic membranes there are often solitary granules appearing like heterophilic granules. The nuclei show some characteristics deviating from those of mammalian plasma cells. They are generally central, comparatively large and with irregular outlines, and they do not show any cartwheel-like arrangement of their heterochromatin, characteristic of mammalian plasma cells. In the assumed plasma cells of *Etmopterus* the heterochromatin is concentrated in a nucleolus-like structure and in a thin frame bordering the nuclear membrane. In some cases the nucleus has a region deviating by a lack of heterochromatin but having a homogeneous appearance. This region may be electron transparent (Fig. 3C) or have a density agreeing with that of the cytoplasmic sacs (Fig. 7).

Discussion

The Leydig organ belongs to the same category of tissues as the epigonal organs of elasmobranchs, the lymphomyeloid tissues of the orbit and the roof of the mouth in holocephalians, and the meningeal lymphomyeloid tissues of chondrosteans and holosteans. These are bone marrow-like tissues which are mainly granulopoietic but also produce lymphocytes.

In *Etmopterus* the Leydig organ is well developed and like the spleen weighs about 0.5% of the body weight (Fänge, 1977). In its microscopical and electron microscopical structure it closely resembles the epigonal organ occurring in many elasmobranchs (Fänge and Mattisson, 1981).

The electron microscope reveals the presence of numerous cells with several characteristics of plasma cells. The cytoplasm of these cells is a structural copy of that from mammalian plasma cells. The characteristic cisternae of endoplasmic

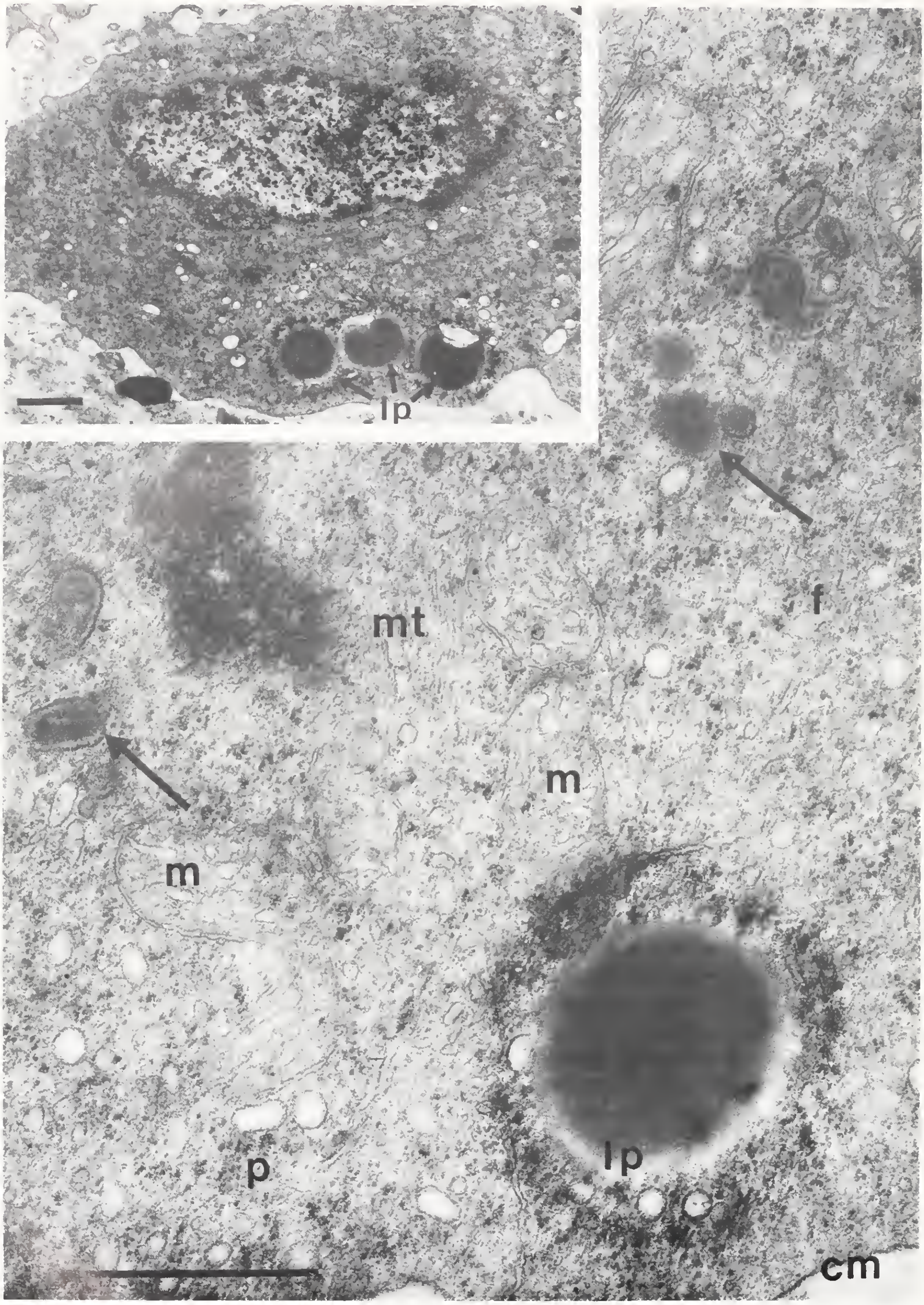

FIGURE 6. Portion of a monocyte-like cell. The cytoplasm contains halo-supplied granules appearing like azurophilic ones (arrows), mitochondria (m), microtubules (mt), polyribosomes (p) and filaments (f). Close to the cell membrane (cm) a large "lipoprotein" granule (lp) surrounded by dense grains. Inset: "Lipoprotein" granules in another monocyte-like cell. Fixation as in Figure 2A. Bars = 1 μm.

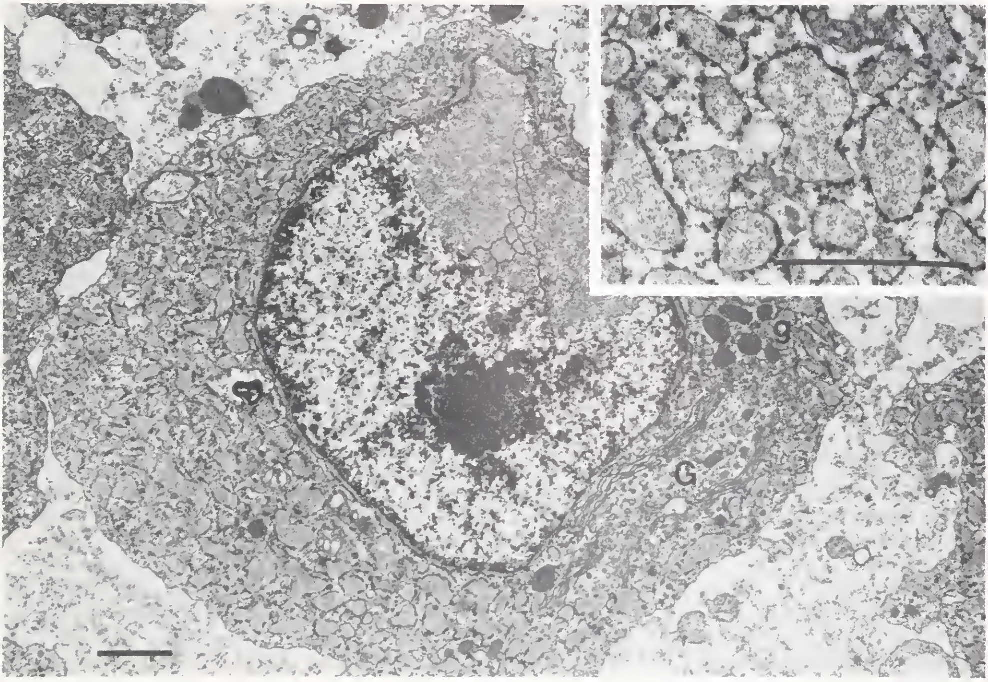

FIGURE 7. Plasma cell. The cytoplasm is filled by rough endoplasmic reticulum in the shape of vesicles containing a moderately dense substance. Close to the Golgi apparatus (G) are some lysosome-like granules (g). In the large nucleus there is a homogeneous moderately dense area divided into smaller portions by delicate membranes. The heterochromatin is centrally localized and forms a thin peripheral frame. Inset: Portion of the cytoplasm showing the ribosomes on the outer part of the vesicular membranes and the moderately dense interior of the vesicles. Fixation as in Figure 2B. Bars = 1 μm.

reticulum are well supplied with ribosomes indicating that the main function of the cells is the synthesis of proteins. The identification of these proteins as immunoglobulins remains to be done. By light microscopy Good *et al.* (1966) identified plasma cells in various lymphomyeloid organs from sharks and rays. Fänge and Mattisson (1981) found plasma cells in the spleen and also in the epigonal organ of the nurse shark, *Ginglymostoma cirratum.* Ellis (1977), without exactly identifying the cell type, observed cells carrying immunoglobulins in the Leydig organ of the ray, *Raja naevus.* Several studies have demonstrated efficient production of immunoglobulins as a response to antigenic stimulation in elasmobranchs (Litman *et al.*, 1976; Marcharlonis, 1977). The presence of great numbers of plasma cell-like cells in the Leydig organ of *Etmopterus* leads us to conclude that this tissue probably has important immune functions.

The electron microscopic studies confirm earlier findings of two types of granulocytes in *Etmopterus* and have contributed further information on the disparity of the granules. In accordance with Fey (1966) we use the term heterophilic for the predominant type of granulocyte. These finely granulated cells may resemble the neutrophils of higher vertebrates, but their granules are eosinophilic rather than neutrophilic. The other main type of granulocyte, here termed eosinophilic, contains large spheric granules which are strongly eosinophilic by light microscopy. The granules differ from those of mammalian eosinophils by their homogeneous electron density lacking crystalline structures. A homogeneous interior of eosinophilic granules was reported by Clawson *et al.* (1966) in the paddlefish (*Polyodon spatula*), a chondrostean fish, by Kélenyi and Németh (1969) in certain primitive fish, and by Morrow and Pulsford (1980) in the dogfish (*Scyliorhinus canicula*).

In addition to the two main types of granulocytes the electron microscope shows cells which appear to be modifications of the heterophilic granulocyte. The discrepancy mainly concerns the granules. Some deviating cells show granules with central translucent vesicles like those described by Kélenyi and Németh from reptiles and amphibia (1969). In other cells most granules appear completely translucent. This structural variation may represent developmental or functional stages. Morrow and Pulsford (1980) likewise report the occurrence of different types of heterophilic granulocytes in the dogfish (*Scyliorhinus canicula*).

The Leydig organ contains numerous large lymphocyte-like cells with distinct nucleoli and abundant cytoplasmic ribosomes. The cellular structure indicates that the majority of these cells may be granuloblasts. As to the small lymphocytes in our electron microscopic study, we have difficulty in distinguishing them from thrombocytes (spindle cells). This difficulty has also been reported for other species of fish by Ellis (1976) and Ferguson (1976).

Several cells show signs of endocytotic activity. Some of these often contain spheric bodies of lipid or lipoprotein density surrounded by electron dense granules (explosions caused by electron bombardment?). These cells also have halo-supplied granules and appear like monocytes or macrophages. However, as asserted by Ellis (1977), the occurrence and designation of monocytes in fishes is confused and contradictory.

Granulocytes and lymphocytes often have pale nuclei pierced by invaginated cytoplasm (Fig. 2E). Yoffey (1980) describes such leptochromatic cells in mammals as "transition cells." The leptochromacia indicates a high activity. In cultures of bone marrow such cells show marked streaming of nuclear material (Rosse, 1971). As to the granulocytes of *Etmopterus* the pale cells are most common among the B- and C-types with their more or less electron lucent granules. These cells often give the impression of being degenerate. The degenerate appearance of leucocytic granules may be due to release of substances from the granules during certain conditions. The substances released could be hydrolytic enzymes, as leucocytic granules from many animals are known to contain a wide array of such enzymes. The Leydig organ of *Etmopterus* is remarkably rich in lysozyme with a high chitinolytic activity (Lundblad *et al.*, 1979; Fänge *et al.*, 1980). *Etmopterus* very commonly are infested by a crustacean parasite, *Anelasma squalicola.* The processes of the parasite, which penetrate deep between skeletal muscle bundles of the shark, are provided with chitinous membranes. Perhaps the chitinolytic enzyme of the Leydig organ, undoubtedly of leucocytic origin, is part of a defense against parasites. However, so far we have not been able to correlate our structural findings such as observations of granulolysis in the leucocytes with the presence of the parasite.

ACKNOWLEDGMENTS

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AGGREGATION AND FUSION BETWEEN CONSPECIFICS OF A SOLITARY ASCIDIAN

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ABSTRACT

Fusion between conspecifics in a solitary ascidian is reported for the first time. *Molgula complanata* Alder and Hancock showed aggregated settlement on Perspex panels in the field, allowing contact between conspecifics after some increase in size. Histological sections of adult animals which were in contact with one or more conspecifics showed that some individuals were fused to others. The frequency of fusion between contacting specimens was 20%. The outer membranes of the tunics were absent between fused animals but present in unfused ones. Fusion was thus characterized by contiguous matrices, which contained cellular elements. No barrier to interchange of tunic cells between fused animals was observed. It is suggested that fusion may oppose inbreeding in hermaphroditic, viviparous ascidians with minimal dispersal.

INTRODUCTION

Fusion of animals derived from separate larvae was first described in 1903 in colonial ascidians. It has since received considerable experimental treatment and is of great interest in the context of comparative immunology (Bancroft, 1903; Hildemann and Reddy, 1973; Tanaka, 1975 for references).

Under experimental conditions fusibility is found in many colonial ascidians (Tanaka, 1975). Fused colonies are indistinguishable from those which are derived through asexual budding. This makes it difficult to study the occurrence of fusion in natural populations (Sabbadin, 1978). It was thought that solitary ascidians, which do not bud, might nevertheless show fusion. Aggregative settlement in solitary ascidians would increase the frequency of contacts between conspecifics and thus increase the chances of possible fusions. However, for ascidians no data exist on the pattern of settlement in the field.

Experiments on the genetic control of colony fusion indicated that fusion may be frequent between closely related conspecifics (Tanaka, 1975 for references; Sabbadin, 1978). In the field, viviparity and a short larval life would be expected to increase the chances of closely related individuals settling near each other. It was therefore decided to examine a natural population of *Molgula complanata*, a viviparous hermaphrodite, for aggregated settlement and for fusion in the resulting clusters.

MATERIALS AND METHODS

The study locality was Langstone Harbor, a fully marine, shallow, natural harbor bordering the northern shore of the eastern Solent (south coast of England).

Perspex settlement panels, 0.25 × 0.25 m square, were fixed to frames and suspended from a raft within 1.5 m of the surface and about 5 m from the seabed.

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To examine aggregated settlement, three horizontally aligned panels were submerged for four weeks during the spring settlement peak (May). The positions of all attached and metamorphosed individuals on the underside of the panels were then determined with the aid of a grid which was scratched onto a thin sheet of a 0.25 × 0.25 m transparent Perspex and placed directly above the settlement panel. The size of the squares of the grid was 10 × 10 mm; the quadrat sizes chosen to test for aggregation were 20 × 20 mm and 40 × 40 mm. The observed distri-

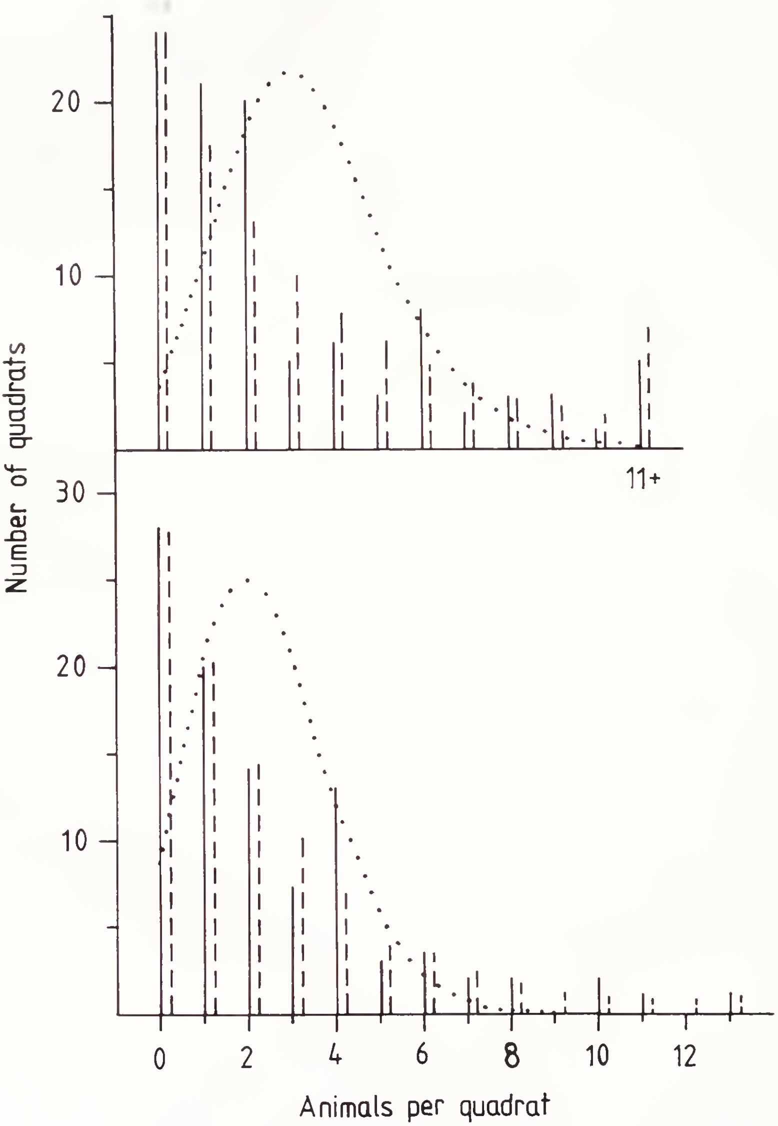

FIGURE 1. Distribution of settled *Molgula complanata* juveniles on 2 replicate panels which had been submerged in the sea for 4 weeks (solid bar). Dotted lines are calculated Poisson distributions; all actual distributions depart significantly from random ($P < 0.001$) but fit well the calculated negative binomial distributions (broken bars).

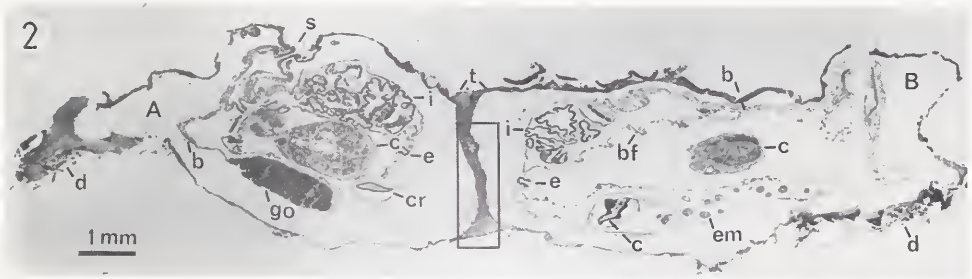

FIGURE 2. Vertical section of *M. complanata* showing extensive fusion over the zone of contact between two individuals, A and B. The framed region is shown at higher magnification in Figure 3. Abbreviations for Figures 2–5: b, body wall; bf, fold of branchial basket; c, associated copepod, *Doropygus pulex* Thorell, in the pharynx; ce, cells in the matrix of the tunic; cr, concretion in renal vesicle; d, debris; e, endostyle; em, embryo; g, gap; go, gonad; i, intestine; om, outer membrane of tunic; s, siphon; t, tunic.

butions were compared with Poisson and negative binomial distributions calculated from the observed data (Ross, 1980).

To examine fusion, aggregated specimens of *M. complanata* were removed from vertically aligned panels which had been submerged for 3–13 months. Individuals in physical contact were carefully separated with forceps, beginning with the side originally attached to the panels. 10 μm vertical sections were made from specimens not separable in this manner. All sections were stained with haematoxylin and eosin.

One side of one panel was analysed for frequency of fusion. Only adult animals ≥ 5 mm were considered.

RESULTS

Aggregation

Comparison of the frequency distribution of recently settled *M. complanata* with expected random (Poisson) distributions verified that settling onto the experimental panels was non-random. All distributions, instead, fitted calculated negative binomial distributions, where the defining parameter 1/k indicates that departures from random were due to individuals being aggregated in groups (Ross, 1980). Data for both quadrat sizes are consistent, and examples of two replicate panels are given in Figure 1 for quadrat size 20 × 20 mm. Although aggregated, the majority of postlarval animals were not in direct physical contact. The percentages of actual contacts between recently settled juveniles were 2%, 2%, and 3% for the three replicate panels.

Other species were present in very low numbers, except the colonial ascidian, *Botryllus schlosseri*, which was as abundant as *M. complanata*. The small colonies of *B. schlosseri*, however, were randomly distributed (*i.e.*, observed distributions fitted expected Poisson distributions) and had apparently not influenced the settlement pattern of *M. complanata*. There was over 95% free space on these panels.

Fusion

The tunics of individuals not separable in the manner described above were fused in the area of contact between animals (Figs. 2, 3, and 4). Cells were abundant in the matrix of the tunics with no evidence of any barrier to cell interchange

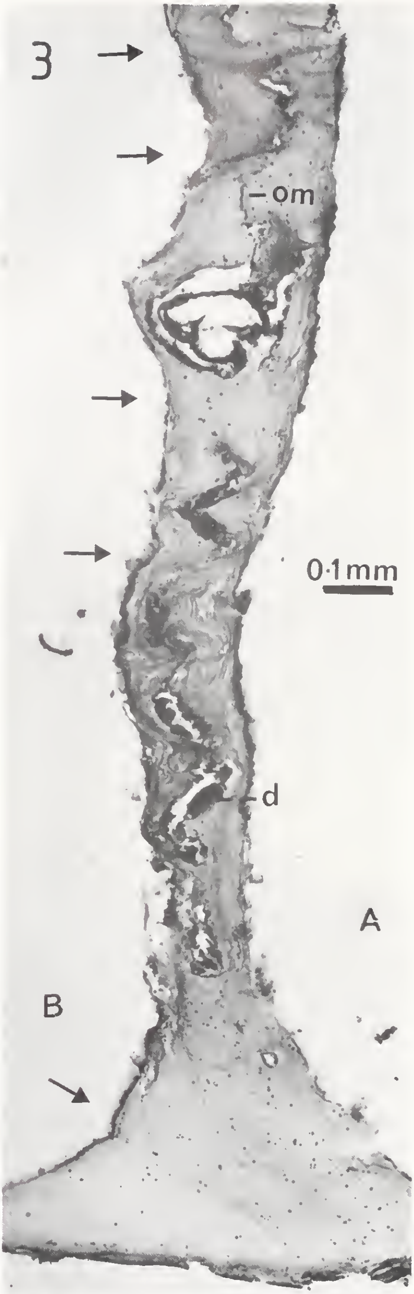

FIGURE 3. Part of the contact region of A and B showing several areas where the tunics are fused (arrows). There are also unfused areas, containing debris and the remains of the outer membranes of the tunics. (Abbreviations: see Figure 2.)

between fused animals. No zones of necrosis were observed. Serial sections showed that fusion could be impeded by debris (Fig. 3). In contrast, Figure 5 shows part of the contact zone between two closely appressed but unfused animals. A line of separation, either as closely adhering outer membranes, or as gaps, is clearly visible.

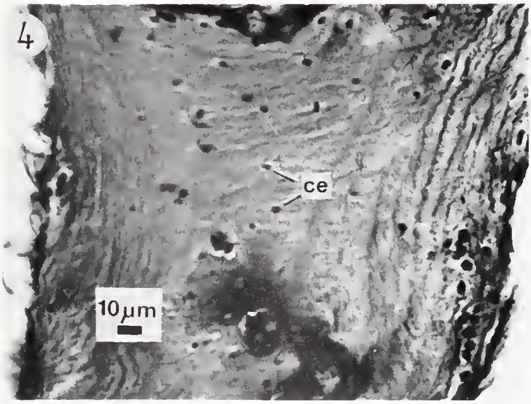

FIGURE 4. Part of Figure 3 at higher magnification. The fibrous matrix is continuous between the fused animals allowing free interchange of the cells contained within the tunic. (Abbreviations: see Figure 2.)

The majority of fusions were between individuals of equal size, but fusion between individuals of several-fold difference in size was also found.

Frequency of fusion

One panel was examined for frequency of fusion. Of 190 animals, 48 were single; occasionally they had unscored juveniles attached to their tunics. The remaining 142 individuals were in direct physical contact with one or more conspecifics, yielding groups of 2–10 animals. As 29 individuals in these groups were unseparable by the method described above, a subsample of 13 of these was analysed histologically. These were fused. It was concluded that about 20% of those which occurred in groups were fused to conspecifics. Fusion was, with one exception, between two ascidians; one group of three fused individuals was found. Although

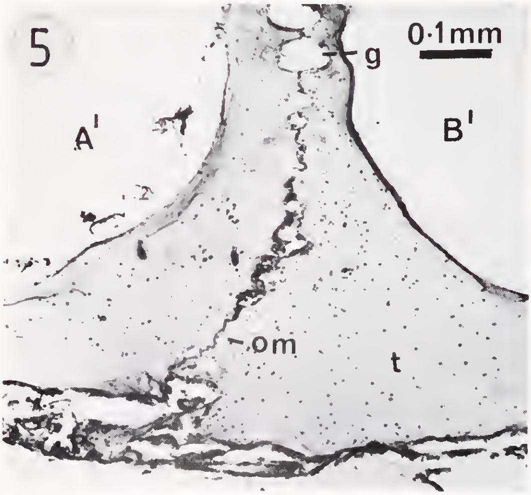

FIGURE 5. Basal part of contact zone of closely appressed but unfused individuals (A′ and B′). A line of division, either as gaps or adhering outer membranes, separates the two animals. (Abbreviations: see Figure 2.)

only one panel was quantitatively sampled for fusion, fused animals were also found on all other panels (11 altogether).

DISCUSSION

This is the first report of aggregated settlement of a solitary ascidian in a natural, field population. There is one account of a laboratory study for another solitary ascidian species which demonstrated aggregative settlement (Young & Braithwaite, 1980). The results indicate that *M. complanata* recognizes conspecifics and settles close to them. The formation of aggregations would favor cross-fertilization in contrast to possible self-fertilization (inbreeding) in this hermaphroditic ascidian. Also, it might have reduced the chances of juvenile *M. complanata* being overgrown by the colonial *B. schlosseri.*

In clusters of adult animals both fused and closely appressed but unfused individuals occurred. Fusion appears to be a relatively frequent, naturally occurring event. This suggests that self versus not-self recognition may exist. This idea successfully explains colony specificity in colonial ascidians (Burnet, 1971; Tanaka, 1975 for references).

Fused animals were apparently compatible. Fusion followed disappearance of the outer membrane of the tunic which is believed to be proteinaceous (Goodbody, 1974). The fibrous matrix of the tunic was continuous between fused individuals, so the cells found in the tunic may have interchanged unimpeded. The tunic cells of ascidians are amoeboid cells and blood cells (Goodbody, 1974). An invasion of tunic cells of an allogeneic animal after tunic fusion might mobilize immune responses, such as those between incompatible colonial ascidians or the cellular reactions between incompatible coelomic cells in solitary ascidians (Tanaka and Watanabe, 1973; Manning and Turner, 1976; Fuke, 1980). But no evidence of any such reactions was found in the present material. For solitary ascidians it has been difficult to obtain information on histocompatibility in laboratory experiments (Hildemann, 1974; Tanaka, 1975).

The life history parameters of *M. complanata* make it likely that closely related individuals, *e.g.* larvae released from the same parent, will occasionally settle in close proximity (*cf.* Sabbadin, 1978; van Duyl *et al.*, 1981) and aggregative settlement would further enhance this tendency. If fused conspecifics were unable to fertilize one another (*c.f.* Sabbadin, 1979), fusion of closely related individuals would promote outbreeding in these populations. This would be of selective value as suggested for fusion in colonial ascidians (Burnet, 1971; Sabbadin, 1978, 1979).

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SODIUM REQUIREMENTS IN HARDENING OF THE FERTILIZATION ENVELOPE AND EMBRYONIC DEVELOPMENT IN SEA URCHINS

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ABSTRACT

The role of sodium ions in fertilization and development of sea urchin (*Arbacia punctulata* and *Strongylocentrotus purpuratus*) eggs was studied by culturing eggs and embryos in low sodium (choline-substituted) sea water. Hardening of the fertilization envelope was impaired in 19 mM Na^+ as indicated by the collapse of this investment 30–60 min after insemination. Cross-linking of the fertilization envelope, assayed in terms of the onset of resistance to dispersal by isotonic urea, was not affected by low sodium. However, impregnation of the fertilization envelope by structural proteins derived from the egg's cortical granules did not take place in low sodium. The "I–T" transition in the configuration of the fertilization envelope in *Strongylocentrotus* from "igloo" shaped casts of microvilli that were at the surface of the unfertilized egg to sharp "tent" shaped spikes also was prevented in low sodium. Potassium and lithium effectively substituted for sodium in promoting the normal structuralization of the fertilization envelope, while choline and Tris did not. Fertilized eggs divided more slowly in low sodium than in normal (419–425 mM Na^+) sea water. *Arbacia* embryos fertilized and cultured in low sodium sea water showed a reversible developmental arrest at the swimming blastula stage. This finding suggests that a sodium activated switch initiates gastrulation (differentiation) in sea urchins. Taken together, these results show that sodium is essential for several physiologic processes related to fertilization and developmental in sea urchins, in addition to the previously described rapid-electrical block to polyspermy and the coupling of early and late events in egg activation.

INTRODUCTION

Sodium, the most abundant cation in sea water, is known to participate in several processes associated with the development of fertilized sea urchin eggs, including: the sperm-initiated electrical depolarization of the egg's plasma membrane (Chambers and deArmendi, 1979), establishment of a rapid block to polyspermy (Jaffe, 1980; Schuel and Schuel, 1981), and the coupled efflux of protons to link the early and late events of egg activation (Chambers, 1976; Epel, 1978).

A possible role for sodium in hardening of the fertilization envelope was suggested by the observation that this investment tended to crenate and collapse in low sodium (choline-substituted) sea water subsequent to its elevation during fertilization (Nishioka and Cross, 1978). The fertilization envelope is derived in part from the vitelline layer that is attached to the plasma membrane of the unfertilized

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egg as well as secretory products discharged by the egg's cortical granules at fertilization (reviewed by Schuel, 1978). Immediately after elevation the fertilization envelope is soft and easily removed by a variety of physical and chemical treatments, but it gradually hardens and becomes extremely difficult to remove or disperse (Kopac, 1940; Harvey, 1956; Lallier, 1971; Veron *et al.*, 1977; Carroll and Baginski, 1978). These changes are promoted by peroxidatic enzymes (Foerder and Shapiro, 1977; Hall, 1978) and structural proteins (Anderson, 1968; Inoue and Hardy, 1971; Chandler and Heuser, 1980) that are secreted by the egg's cortical granules (Schuel, 1978).

The present communication defines the functional role of sodium ions in hardening of the fertilization envelope, and also identifies sodium requirements for cell division and for gastrulation during subsequent embryonic development in sea urchins. Preliminary accounts of portions of this study have been presented previously (Schuel *et al.*, 1979, 1981).

MATERIALS AND METHODS

Gametes from the sea urchins *Arbacia punctulata* and *Strongylocentrotus purpuratus* were used in this study. *Arbacia* were collected locally at Woods Hole, MA during the summer months and kept in fresh running sea water. During the winter *Arbacia* (obtained from Florida Bio-specimen Co., Panama City, FL) and *Strongylocentrotus* (obtained from Pacific Bio-Marine Supply Co., Venice, CA) were kept at Buffalo in a marine aquarium (Aquarium Systems, Inc., Wickliffe, OH).

Gametes were obtained by injection of isotonic (0.5 M) potassium chloride into the coelomic cavity of adult sea urchins (Harvey, 1956). Semen was collected "dry" and stored over crushed ice. Working sperm suspensions were prepared by diluting the semen with sea water just prior to insemination. The eggs were collected in sea water, filtered through cheese cloth, washed with sea water, and stored over crushed ice until used. The eggs were inseminated and cultured at 24°C for *Arbacia* and 15°C for *Strongylocentrotus*. The culture media consisted of either natural (425 mM Na^+) sea water (Cavanaugh, 1964), artificial (419 mM Na^+) sea water (Goldstein, 1953), or low sodium (19–26 mM) sea water prepared by equimolar substitution of choline chloride for sodium chloride (Chambers and deArmendi, 1979). In certain experiments potassium, lithium, and Tris (tris(hydroxymethyl)aminomethane) were used as sodium substitutes in place of choline (Gould-Somero *et al.*, 1979). Since sea urchin eggs will not fertilize in potassium- or lithium-substituted sea water, they were fertilized in normal artificial sea water and transferred into these solutions at 10 sec after insemination. At this time essentially all the eggs had been activated, while less than 10% of the cortical granules had undergone exocytosis (Dandekar and Schuel, unpublished data). Choline chloride and Tris were obtained from Sigma Chemical Co. (St. Louis, MO).

Hardening (cross-linking) of the fertilization envelope was evaluated on the basis of the onset of resistance to solubilization in isotonic (1.0 M) urea (Moore, 1930; Kopac, 1940). The eggs (0.4 ml) were pre-incubated for 5 min in artificial or low sodium sea water (10 ml) and then inseminated by addition of 0.1 ml of 10% sperm. The eggs were removed from the cultures by gentle centrifugation (IEC Clinical Centrifuge, head #221 at setting 5 × 20 sec) at various times post insemination, washed (2 × for 60 sec) in 10 ml of 1.0 M urea, returned to sea water, and scored for the presence of the fertilization envelope as observed by light microscopy.

Cleavage time was determined by fixing the cultures with 2% glutaraldehyde at various intervals before, during, and after first cleavage. The eggs were examined by light microscopy and divided zygotes were counted. The time at which 50% of the population had divided was estimated from plots of percent divided *vs* time.

Approximately 100–200 eggs were counted in each culture. The data are presented as mean values plus or minus the standard deviation for the indicated number (N) of experiments. The statistical significance between control and experimental eggs was evaluated on the basis of the "*t*" test for two sample means (Brownlee, 1965).

Ultrastructural observations were performed on eggs fixed and processed for transmission electron microscopy using previously described procedures for *Arbacia* (Longo and Anderson, 1972) and *Strongylocentrotus* (Summers and Hylander, 1974).

RESULTS

The fertilization envelope elevated from *Arbacia* and *Strongylocentrotus* eggs within 60 sec after insemination in both normal (419–425 mM Na^+) and sodium-depleted (19–26 mM) sea water. In low sodium (choline-substituted) sea water the perivitelline space appeared to be wider, and the fertilization envelope appeared to be thinner and more refractile as observed by light microscopy. The fertilization envelope normally thickens and becomes darker within several min post insemination in normal sea water. However, in low sodium the fertilization envelope remained thin and refractile, and by 15 to 45 min after insemination it began to crenate and collapse onto the surface of the zygote (Fig. 1). Transmission electron microscopy revealed that cortical granule exocytosis was completed by 60 sec after insemination in both normal and sodium-depleted sea water (see Fig. 4A, below). There was no indication that any of the components released by the cortical granules had been precipitated by the choline-substituted sea water. Taken together these observations confirmed the previous findings by Nishioka and Cross (1978) that hardening of the fertilization envelope was impaired in low sodium sea water, and suggested that some process subsequent to the release of the contents of the cortical granules had been affected. Accordingly, we studied the effects of sodium-depleted sea water on two later manifestations of fertilization envelope hardening in sea urchins: structuralization by released cortical granule contents (Anderson, 1968; Inoue and Hardy, 1971), and the onset of reduced solubility in isotonic urea (Kopac, 1940). The latter is a measure of the extent of intermolecular cross-linking (Lorand, 1972).

Transmission electron microscopic observations showed that the gradual structuralization of the fertilization envelope did not take place in choline-substituted low sodium sea water (Figs. 2 and 3). A thin and fluffy fertilization envelope was present in *Arbacia* (compare Figs. 2A and 2B) and *Strongylocentrotus* (compare Figs. 3A and 3B) eggs at 60 sec post insemination in both normal and low sodium sea water. One hour later a thickened tri-laminar fertilization envelope, typical of this matured and fully hardened external investment (Anderson, 1968; Inoue and Hardy, 1971), had developed in normal (419–425 mM Na^+) sea water (Figs. 2C and 3C). However, in low sodium (19–26 mM) the morphology of the fertilization envelope at one hour had not changed from that observed at 60 sec (compare Figs. 2B and 3B with 2D and 3D). The fertilization envelope in *Strongylocentrotus* (Figs. 3A and 3B), but not in *Arbacia* (Figs. 2A and 2B), initially retains rounded "igloo or I" shaped casts of the microvilli that were at the surface of the unfertilized egg. These casts in *Strongylocentrotus* gradually become "tent or T" shaped as the

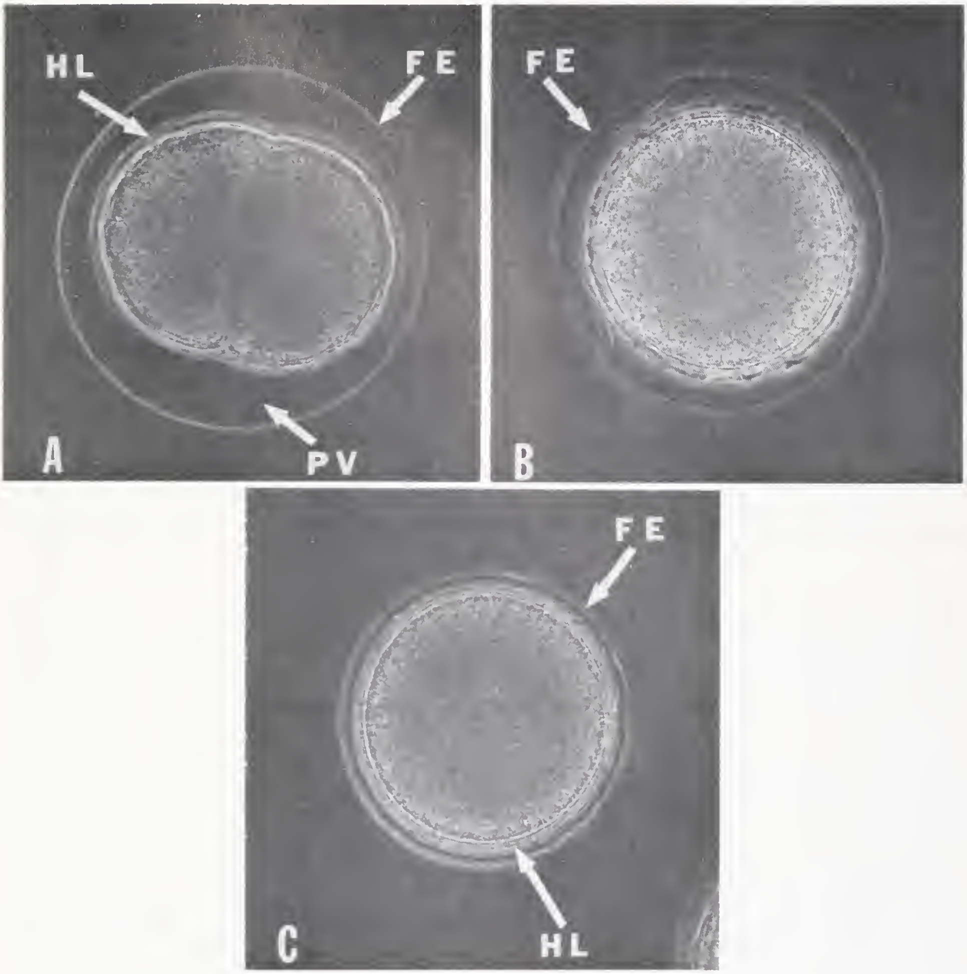


FIGURE 1. Collapse of fertilization envelope in *Strongylocentrotus purpuratus* eggs induced by sodium-depleted (choline-substituted) sea water. Phase contrast micrographs of living eggs photographed at time of first cleavage in 412 m*M* Na^+ sea water (A). Eggs cultured in 19 m*M* Na^+ sea water (B, C) have not begun to divide at this time. Mag. 440 ×.

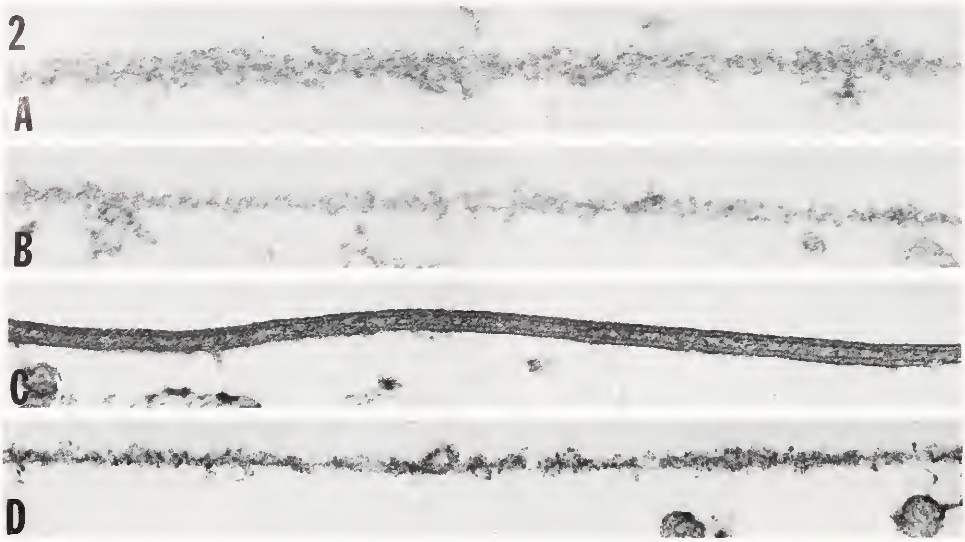
(A): Note the spherical shape of the normally hardened fertilization envelope (FE), fully developed perivitelline space (PV), and the hyaline layer (HL) that is applied to the surface of the dividing zygote cultured in normal sea water.

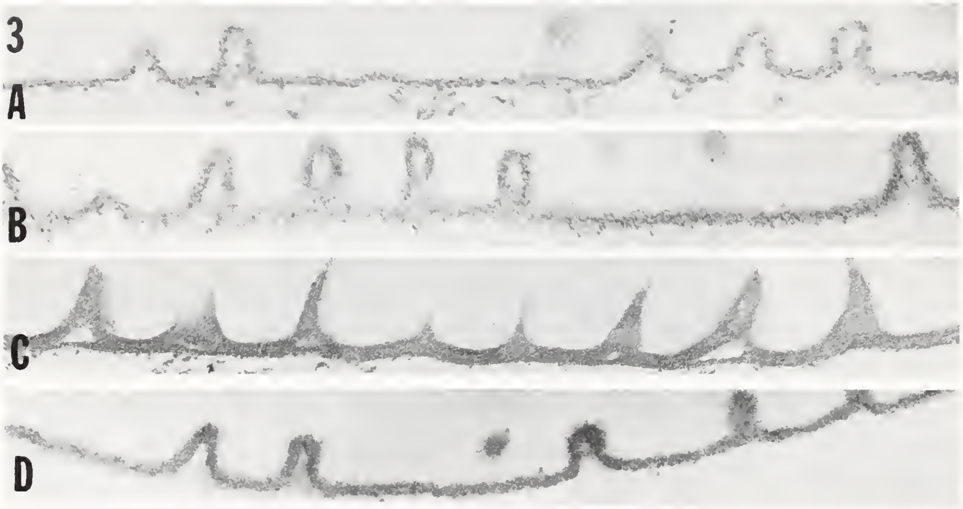
(B): The fertilization envelope is crenated and partially collapsed in this zygote cultured in low sodium sea water.

(C): The fertilization envelope has completely collapsed onto the hyaline layer in this low sodium sea water treated zygote.

fertilization envelope hardens and structuralizes (Veron *et al.*, 1977). This "I–T" transition in *Strongylocentrotus* does not take place in low sodium (choline-substituted) sea water (compare Figs. 3C and 3D). Similar effects were observed with Tris-substituted low sodium sea water (data not shown). However, when potassium or lithium were used to replace sodium instead of choline the fertilization envelope structuralized normally and did not collapse (data not shown).

Cross-linking of the fertilization envelope, assayed by the loss in urea solubility,





FIGURES 2 and 3. Transmission electron micrographs showing the effects of low sodium (choline-substituted) sea water on structuralization of the fertilization envelope in *Arbacia* (Fig. 2) and *Strongylocentrotus* (Fig. 3). Panels A: normal sea water at 60 sec post insemination: Panels B: low sodium sea water at 60 sec; Panels C: normal sea water at 60 min; Panels D: low sodium sea water at 60 min post insemination. In all micrographs the perivitelline space is at the bottom. Mag. Fig. 2, 64,000 ×; Fig. 3, 40,000 ×.

was not affected by low sodium sea water (Table I). The data show that all the fertilization envelopes were solubilized by 1.0 *M* urea at 1 min after insemination, about half were soluble at 5 min, and all were insoluble by 10 min in both artificial (419 m*M* Na^+) and 19 m*M* Na^+ (choline-substituted) sea water. The removal of the soft fertilization envelope by urea was confirmed by transmission electron microscopy (Fig. 4). Both the fertilization envelope and hyaline layers were completely removed from eggs inseminated in 19 m*M* Na^+ and treated with urea at 60 sec

TABLE 1

Effect on sodium-depleted (choline-substituted) sea water on hardening of fertilization envelope in S. purpuratus.

Treatment	Fertilized (% FE)	Urea insoluble fertilization envelopes (%) at min post insemination 1	5	10
Sea water ($412\ mM\ Na^+$)	100.0 ± 0.0	0.0 ± 0.0	42.0 ± 16.0	98.9 ± 1.5
Sodium-depleted SW ($19\ mM\ Na^+$)	100.0 ± 0.0	0.0 ± 0.0	59.3 ± 24.0	98.0 ± 2.8
			($P < 0.2$)	($P > 0.5$)

Experimental conditions described in text under Methods.
N = 4.

(Figs. 4A and 4B). When eggs were treated with urea at 10 min after insemination both the fertilization envelope and hyaline layers remained intact (Figs. 4C and 4D). Similar results were obtained with eggs fertilized in 419 $mM\ Na^+$ (data not shown). There was no morphological difference in the development of the hyaline layers in eggs fertilized in normal and sodium-depleted sea water. The hardening of the hyaline layer is known to be promoted by the formation of cross-links via calcium ions (Citkowitz, 1971).

Low sodium (choline-substituted) sea water also was observed to retard cleavage (see Fig. 1, above) and prevent normal embryonic development. Previous studies showed that sea urchin eggs are extremely vulnerable to polyspermy in sodium-depleted sea water (Jaffe, 1980; Schuel and Schuel, 1981). Furthermore, polyspermic eggs are known to divide more slowly than monospermic zygotes (Schuel *et al.*, 1973; Longo *et al.*, 1974) and rarely develop beyond the blastula stage (Morgan, 1927). Hence, these experiments were conducted with eggs that had been inseminated with a minimal sperm density just sufficient to fertilize all the eggs in low sodium without inducing significant polyspermy.

The retardation of first cleavage in eggs fertilized and cultured in sodium-depleted sea water was quantitatively documented in *Arbacia* (Fig. 5). In three similar experiments the time for 50% of the eggs to divide in natural (425 mM Na^+) sea water was 48.2 ± 2.8 min, compared to 62.0 ± 1.3 min in 26 $mM\ Na^+$ at $24.3 \pm 0.6°C$ ($P < 0.02$). The time required for first cleavage of control eggs in this study is consistent with previous observations on *Arbacia* under similar conditions (Harvey, 1956). The delay in first cleavage induced by sodium-depleted sea water in these experiments was 13.8 ± 3.3 min.

Early development of *Arbacia* zygotes continued in low sodium (26 mM) sea water, but was arrested at the swimming blastula stage. This effect was completely reversible and development into mature larvae (plutei) ensued if the embryos were returned to natural sea water anytime between 30 sec to 24 hours after insemination. By 24 hours after insemination in either natural or artificial (425 $mM\ Na^+$) sea water, gastrulation had been completed and young plutei were actively swimming in the cultures. If 24 hours old *Arbacia* embryos arrested at the swimming blastula stage in 26 $mM\ Na^+$ were returned to natural or artificial (425 $mM\ Na^+$) sea water at this time, they resumed development and reached the pluteus larva stage within another 24 hours.

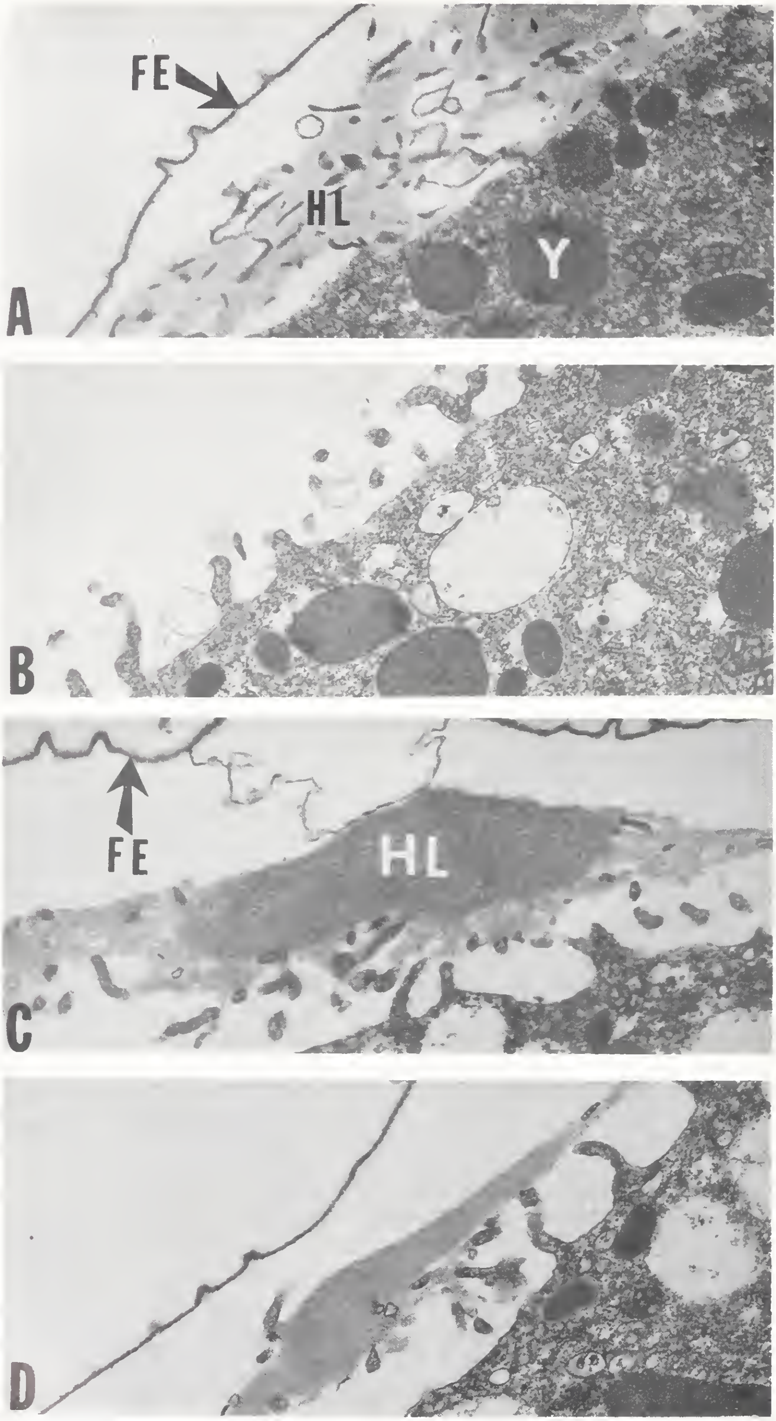


FIGURE 4. Transmission electron micrographs of *S. purpuratus* eggs fertilized in 19 mM Na^+ (choline-substituted) sea water and treated with 1.0 M urea to remove the fertilization envelope as described in Table I. Mag. 18,000 ×.

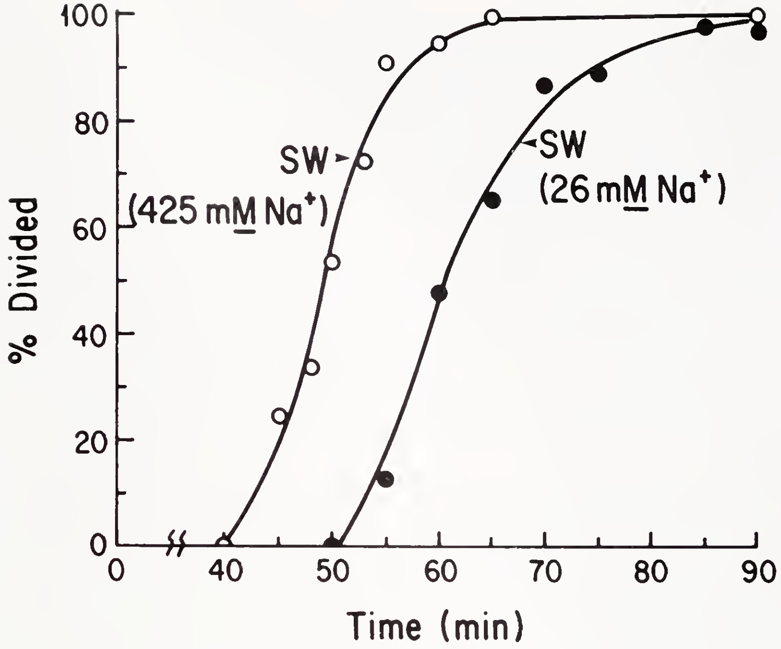


FIGURE 5. Delay in first cleavage of *Arbacia* eggs induced by sodium-depleted sea water. Eggs inseminated with minimal sperm, cultured at 24°C, and fixed at indicated times post insemination.

DISCUSSION

The importance of the common cations in sea water in regulating processes associated with fertilization and embryogenesis in sea urchins has been recognized for many years (see reviews by: Herbst, 1904; Loeb, 1913; Lillie, 1919; Heilbrunn, 1956; Runnstrom, 1966; Epel, 1978; Schuel, 1978). The results of the present study identify additional roles for sodium ions in these phenomena.

Elevation of the vitelline layer from the sea urchin egg surface and its gradual transformation into the fully hardened fertilization envelope is a complex process that is mediated by the secreted products of the cortical granules plus cations in the ambient sea water. Detachment of the vitelline layer from the egg's plasma membrane is promoted by a cortical granule derived serine protease (Longo and Schuel, 1973; Schuel *et al.*, 1973; Carroll, 1976) which requires calcium to maintain its enzymatic activity (Vacquier, 1975). Elevation of the detached vitelline layer is promoted by the secretion of acidic polyanions and other cortical granule derived macromolecules into the perivitelline space (Schuel *et al.*, 1974; Schuel, 1978; Green and Summers, 1980). The subsequent hardening of the fertilization envelope is promoted by other released cortical granule products: a peroxidase which cat-

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(A): Egg fixed at 60 sec after insemination. Fertilization envelope (FE), hyaline layer (HL), yolk platelet (Y). Cortical granules have all undergone exocytosis by this time. Degranulation completed by 60 sec in normal (419 m*M* Na$^+$) sea water (data not shown).

(B): Egg treated with 1.0 *M* urea at 60 sec after insemination. Note the complete removal of fertilization envelope and hyaline layer.

(C): Egg fixed at 10 min after insemination.

(D): Egg treated with 1.0 *M* urea at 10 min after insemination. The fertilization envelope and hyaline layers are intact.

alyzes the formation of dityrosine cross-links (Foerder and Shapiro, 1977; Hall, 1978), and structural proteins that are inserted into the fertilization envelope (Anderson, 1968; Inoue and Hardy, 1971; Chandler and Heuser, 1980). The results of the present study indicate these are separate events in the hardening process. Structuralization appears to be sodium dependent, while cross-linking is not.

The increased resistance of the fertilization envelope to dissolution by urea, as well as other organic dispersing solvents (Lallier, 1971; Foerder and Shapiro, 1977; Carroll and Baginski, 1978; Hall, 1978), during hardening is analogous to that associated with the polymerization of fibrin during the coagulation of mammalian blood (Lorand, 1972). In both systems the resistance to solubilization by 1.0 *M* urea reflects the formation of new covalent bonds to cross-link a previously soft and easily dispersed structure. The results of the present study are consistent with this hypothesis since the urea treatment completely removed the fertilization envelope at 1 min post insemination but was ineffective if applied at 10 min. Our data also show that this transition is not impaired in low sodium (19 m*M*) sea water.

The fertilization envelope normally is impregnated by cortical granule derived structural proteins to form a thickened tri-laminar investment (Anderson, 1968; Inoue and Hardy, 1971; Chandler and Heuser, 1980). Polymerization of the paracrystalline protein that contributes to the structuralization of the fertilization envelope depends upon the divalent cations, calcium and magnesium, found in sea water (Bryan, 1970). Isolated soft (non-cross-linked) fertilization envelopes undergo a "wraithing" process, altered refractility and loss in rigidity, in low sodium media (Kay *et al.*, 1980) which mimics those observed by us *in vivo*. Our results suggest that the actual insertion of cortical granule derived structural proteins into the fertilization envelope as well as the "I–T" transformation are sodium-dependent processes. Potassium and lithium can effectively replace sodium in promoting these structural changes, while choline and Tris can not. Alternatively it may be the presence of choline or Tris rather than absence of sodium that is responsible for the observed defects in assembly of the fertilization envelope. In either case choline- and Tris-substituted sea water should be a useful tool to probe the mechanism for insertion of the cortical granule derived structural proteins into the fertilization envelope during hardening.

The "I–T" transformation of the *Strongylocentrotus* fertilization envelope appears to depend upon the inserted structural proteins. This notion is supported by morphological evidence that these processes take place concurrently during fertilization envelope hardening (Chandler and Heuser, 1980), and by the observation that removal of these structural proteins from the hardened fertilization envelope *in vitro* by repeated extraction with 6.0 *M* urea-1.5 *M* mercaptoethanol (pH 10) at 100°C results in the disappearance of the "T" projections (Carroll and Baginski, 1978). The fertilization envelope in *Arbacia* does not retain casts of microvilli that were at the surface of unfertilized egg and thus does not undergo an "I–T" transformation, but it does structuralize during hardening. This implies a species difference in the properties of the vitelline layer and fertilization envelope between *Arbacia* and *Strongylocentrotus*. However, the rigidity of the hardened fertilization envelope appears to depend upon the inserted structural proteins in both species. Failure of this process in low sodium sea water may account, in part, for the observed crenation and collapse of the fertilization envelope.

Subsequent to these changes, the permeability of the fertilization envelope to proteins gradually is restricted (Veron *et al.*, 1977). This alteration in the fertilization envelope probably prevents the escape of macromolecules from the perivitelline space, and also may act to further insulate the developing embryo from potential hazards in its environment. The hydration of colloidal polyanions that are secreted by the egg into the developing perivitelline space during fertilization is believed to play a major role in the initial elevation of the fertilization envelope (Schuel, 1978). If the restricted permeability of the fertilization envelope does not develop normally in low sodium sea water, then the gradual loss of colloidal polyanions from the perivitelline space could contribute to the collapse of the fertilization envelope.

The cleavage delay and the reversible failure to gastrulate in low sodium (26 m*M*) sea water are processes distinct from the previously documented requirement for a minimum of 3 m*M* Na^+ for the coupled efflux of protons during activation of the egg at fertilization in order to initiate cleavage and development (Chambers, 1976; Epel, 1978). If eggs are transferred to zero sodium sea water at 30 sec after insemination, development of the incorporated male pronuclei, sperm aster formation, fusion of male and female pronuclei, and cell division are suppressed (Carron and Longo, 1980). It is unlikely that such a defect in pronuclear development is responsible for the delay in the first cell division observed in the present study, since fusion of male and female pronuclei takes place at the same time in 19 m*M* Na^+ as in eggs inseminated and cultured in normal sea water (Schuel and Schuel, 1981). Additional work is required to identify the lesion responsible for the delay in cell division promoted by low sodium (19–26 m*M*) sea water.

Developmental arrest of sea urchins at the swimming blastula stage in sodium-depleted sea water (composed of magnesium, potassium, and calcium salts) was first reported by Herbst (1904). More recently Chambers (1976) observed that eggs cultured in choline-substituted low sodium sea water developed into blastulae, but he did not look for effects on later stages of development (Chambers, personal communication). The results of the present study confirm and extend these previous observations by showing that development will resume when arrested blastulae are returned to sea water that contains normal levels of sodium. Other kinds of treatments which produce developmental arrest at the swimming blastula stage in sea urchins prevent the activation of genes which direct the synthesis of new proteins required for differentiation (Gross, 1967). Sodium ions have been reported to selectively activate genes and induce chromosomal puffing in other systems (Lezzi, 1970). Taken together these findings suggest that a sodium-activated switch may regulate similar processes essential for gastrulation in sea urchins. Alternatively, the sodium switch may operate at the translation or post-translation levels. Additional work is required to answer these questions.

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OBLIGATE AND FACULTATIVE SUSPENSION FEEDING IN ANURAN LARVAE: FEEDING REGULATION IN *XENOPUS* AND *RANA*

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Abstract

The obligate suspension-feeding *Xenopus laevis* larva can survive solely on particles over a wider concentration range, but the facultative suspension-feeding *Rana pipiens* complex larva has a greater behavioral plasticity in its feeding. Regulatory mechanisms for adjusting filtering and ingestion rates reflect the morphological differences between these tadpoles. *Xenopus* larvae regulated feeding primarily by altering buccal pumping rate and *Rana* by adjusting buccal volume. The feeding data for both species fitted a rectangular hyperbola, with a lower concentration "threshold," and were consistent with most predictions of "energy-optimization" models. The significance of a feeding "threshold" is discussed. Regulation by satiation may be a fundamental universal regulatory mechanism for feeding in phylogetically-diverse suspension feeders. Negative allometry for buccal volume *vs* snout–vent length was noted for *Rana* and *Xenopus* larvae. Over all size classes, buccal volumes of *Rana* were smaller than those for *Xenopus*, confirming conclusions from earlier morphological studies. Over evolutionary time, the energetic consequences of different rates of supply of particles in temperate (*Rana*) *vs* tropical (*Xenopus*) waters, among other factors, may have selected for the observed differences in these tadpoles' feeding abilities. A number of behavioral adjustments, such as migration, metamorphosis, threshold feeding, and prey switching, may serve to minimize overexploitation of the prey of vertebrate suspension feeders, while insuring adequate food to supply energetic needs.

Introduction

The most abundant and productive food source in aquatic ecosystems, microscopic suspended particles, generally can be exploited directly only by suspension feeders. A diversity of adaptations enable representative species from virtually all animal classes to entrap such particles (Jørgensen, 1966). However, analyses of this critical process have focused on zooplankton, numerically the most abundant suspension feeders. Comparative studies, in vertebrates as well as invertebrates, could lead to a more general understanding of the process of suspension feeding, its evolutionary significance, and its potential influence on planktonic organisms.

Anuran larvae are among the more versatile and efficient of the vertebrate suspension feeders, ingesting particles with diameters < 0.2 μm (Wassersug, 1972) to >200 μm (Seale, 1980). The feeding dynamics of tadpoles are similar to those of zooplankton (Seale and Wassersug, 1979; Seale and Beckvar, 1980; Seale *et al.*, 1982). For any suspension feeder, at a given particle concentration, C, the filtering rate, F, is the volume of water cleared, and ingestion rate, I, is the corresponding

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Abbreviations: *BV*, buccal volume; *C*, particle concentration; *CC*, critical concentration; *F*, filtering rate; *I*, ingestion rate; *P*, pumping rate; *SV*, snout to vent length; *TC*, threshold concentration.

amount of food thus obtained, per unit time (see Mullin *et al.*, 1975). Mechanisms for adjusting *F* and *I* can be deduced from the tadpole's feeding process. Water is brought into the buccal cavity with a buccal pump; suspended particles are captured on gill filters and by a mucus-entrapment mechanism (DeJongh, 1968; Kenny, 1969; Gradwell, 1972a, b; Wassersug, 1972, 1980; Wassersug and Hoff, 1979). Hence, tadpoles can regulate *F* and *I* by adjusting: 1) pumping rate, 2) volume of water cleared per pump stroke (*i.e.* the buccal volume), and/or 3) entrapment efficiency (Seale and Wassersug, 1979; Wassersug and Hoff, 1979; Wassersug and Rosenberg, 1979; Seale *et al.*, 1982).

Anuran larvae offer a unique opportunity to compare mechanisms for feeding regulation in facultative *vs* obligate suspension feeders, within the same taxonomic order. With a few exceptions, the larvae of pipid frogs, such as the genus *Xenopus*, are obligate suspension feeders, whereas larvae of non-pipid frogs, such as the genus *Rana*, are facultative suspension feeders (Gradwell, 1971, 1972a, b, 1975; Wassersug and Hoff, 1979; Wassersug, 1980). In preliminary studies, obligate suspension-feeding *Xenopus* larvae regulated *F* and *I* primarily by varying buccal pumping rate and *Rana* larvae mostly by adjusting buccal volume and to a lesser extent by adjusting pumping rate (Seale and Wassersug, 1979; Wassersug and Hoff, 1979; Seale *et al.*, 1982). Morphological differences in these larvae were consistent with experimental results (Seale and Wassersug, 1979; Wassersug and Hoff, 1979; Seale *et al.*, 1982), providing a partial confirmation of these differences in feeding regulation. However, the experiments could be criticized in some respects. First, the comparisons were not made under identical conditions: 1) *Rana* larvae were field collected but *Xenopus* larvae were laboratory raised; 2) *Rana* larvae were conditioned longer than *Xenopus* larvae; and 3) the containers allowed for settling differences, *e.g.* between experimental and control vessels. Secondly, earlier studies used *R. sylvatica*, an atypical *Rana* larva. Most *Rana* larvae are characteristically benthic, whereas *R. sylvatica* larvae have morphological traits more commonly seen in midwater larvae which are primarily adapted for entrapping planktonic organisms (Wassersug and Hoff, 1979).

Here I describe experiments incorporating improvements in methodology to examine regulation of feeding in the larvae of *Xenopus laevis*, the South African clawed frog, and of two species in the *Rana pipiens* complex, leopard frogs, representing "typical" *Rana* tadpoles (Wassersug and Hoff, 1979). In most respects, the data confirmed previous findings (see above). Results are discussed in relation to predictions and assumptions of general models of suspension feeding, and to the potential effects and evolutionary significance of vertebrate suspension feeders in aquatic ecosystems.

Materials and Methods

Suspension-feeding dynamics

Anuran larvae were raised from fertilized eggs in the laboratory. *Rana pipiens* adults were purchased by J. Turpen from the Lemberger Co., in Wisconsin. The species identification was verified from adult characters (Pace, 1974). *Xenopus laevis* larvae were obtained from E. Hibbard. The *X. laevis* larvae were fed solely on yeast, *Saccharomyces cerevisiae*, the experimental food. The *Rana* larvae were raised on commercial fish food, but were fed exclusively on yeast suspensions for at least 3 weeks prior to experiments. All tadpoles were in Gosner (1960) stage 40 or earlier.

As an improvement over previous experimental design (Seale and Wassersug, 1979; Seale and Beckvar, 1980; Seale, *et al.*, 1982), the grazing chambers incorporated a circulating sidearm (modified from Řičica, 1966), which minimized differential settling between experimental and control chambers, and between the two species of larvae. The chambers were constructed from 250 ml plastic, screw-capped, inverted Erlenmeyer flasks, with their normal bottoms removed. A screen was placed diagonally about 0.5 cm above the edge of the screw cap in each flask; this barrier prevented the tadpole from swimming into the flask's neck, and isolated settled fecal matter from the animal. A "Y"-tube sidearm was attached to two 3 mm I.D. plastic tubes inserted into each flask; one was about 1 cm above the screen, and the other was vertical above the first and about 1 cm below the open top. The base of the "Y" tube was attached through vinyl tubing to an aeration pump. Activation of the pump caused water to circulate from the bottom of the chamber through the sidearm, and then to exit with a gentle pulsatile action through the top tube. These modifications allowed circulation and aeration of the yeast without producing strong currents or bubbles within the chambers.

Tadpoles were acclimated to experimental conditions and prefed on yeast for at least 24 h prior to each experiment. Within 2 h of each experiment, fresh yeast suspensions were made from dried, commercial yeast in dechlorinated tap water. Three experimental (one tadpole each) and one control (no tadpole) chambers were monitored simultaneously. Experiments were conducted in the light at 20.0 ± 0.5°C for 4–10 h, beginning in early afternoon. Data collection was delayed for 1 h because after handling, respiration (Feder, 1981) and feeding (Wassersug and Hoff, 1979; Seale and Beckvar, 1980) rates were erratic for at least 45 min in earlier studies. Mean pumping rate (number of buccal depression $\cdot \mathrm{min}^{-1}$) was obtained from at least 6 independent visual estimates on each animal at sampling time, using a stopwatch. After measuring pumping rates, but before taking yeast samples, I scraped the interior of each chamber with a rubber rod, using extreme caution not to disturb the animal. Samples from the chambers were processed for counting, and biovolumes were standardized, as previously described (Seale and Wassersug, 1979; Seale and Beckvar, 1980).

An electronic particle-counting system was used to enumerate and concurrently to obtain a total biovolume (wet cell volume) for each yeast sample (see below). The system incorporated several improvements over the one used in earlier studies (see Seale and Wassersug, 1979; Boraas, 1980; Seale and Beckvar, 1980). It consisted of a Model ZB Coulter Counter with external manometer stand, interfaced with a C-100 Channelyzer through a modified Coulter teleprinter interface to a microcomputer. The modifications needed to route signals from a Channelyzer through a Teleprinter interface to a microcomputer, rather than a teletype, are relatively simple; the design and software for our system is available upon request (see acknowledgments). This system allowed for rapid data collection and storage for later manipulations, as well as immediate on-line computation of total number and biovolume. Transmission errors were detected by comparing the Channelyzer reading to the computer readout for number. The Coulter Counter was subject to inaccuracies at C below $5\text{–}9 \times 10^4\ \mu\mathrm{m}^3 \cdot \mathrm{ml}^{-1}$ because of background. Electronic noise contributed to this background, which varied from day to day; this is a source of error in all electronic particle-counting systems.

The tadpole filtering rate, F, was computed indirectly from yeast concentration, and ingestion rate was computed as FC, as previously described (Seale and Wassersug, 1979; Seale and Beckvar, 1980). The mean yeast concentration, C, was estimated as $(C_t - C_0)/\ \ln\ (C_t/C_0)$, where C_0 is the yeast concentration at the beginning and C_t that at the end of the sample interval. This indirect method for estimating filtering rate assumes an exponential decline for C during each sampling interval, and 100% efficiency of particle retention, which may not be valid under all conditions (see Coughlan, 1969; Seale and Wassersug, 1979; Seale and Beckvar, 1980; Seale *et al.*, 1982). Tadpole buccal volumes were estimated by dividing filtering rate by pumping rate for each time interval. Since the tadpoles' responses to C might be affected by the amount of time they had spent in the chambers, the initial C_0 was varied for each experiment. After at least 2 sampling intervals, C was raised by adding a portion of a 1% yeast suspension and the chamber sampled for at least one more time interval. Based on previous results (Seale and Wassersug, 1979), C was expressed as biovolume $\cdot \text{ml}^{-1}$ and I was computed in terms of (μm^3 of yeast) $\cdot$ (tadpole g wet mass)$^{-1} \cdot \text{h}^{-1}$.

Parameter estimates for nonlinear equations were obtained by nonlinear regressions on the data as previously described (Seale and Beckvar, 1980; Seale *et al.*, 1982). Parameter estimates were selected after an examination of standardized residual plots (Neter and Wasserman, 1974).

In this paper, all errors are presented as ±95% confidence intervals.

Buccal volume as a function of individual size

Upon the completion of the feeding studies described above, I examined the relationship between individual size and apparent buccal volume for larvae of *X. laevis* and of *R. pipiens* complex in more detail. These studies were initiated in late spring, when *R. pipiens* adults no longer were available for laboratory fertilization. I obtained a fresh clutch of *R. sphenocephala* (= *R. utricularia*; see Pace, 1974), the southern leopard frog, from the Pine Barrens near Hammonton, New Jersey. This is the only leopard frog in this region (Conant, 1975).

Both the *Rana* and the *Xenopus* larvae were raised on yeast from hatching. Individuals from each of these two growing cohorts were selected at random on dates of experiments. All tadpoles were in Gosner (1960) stages 26–40. Experiments were conducted as described above, except that the animals were allowed to acclimate for at least 2 h before sampling, and only two intervals were sampled for each tadpole. The narrow concentration range examined ($3\text{–}6 \times 10^7\ \mu\text{m}^3 \cdot \text{ml}^{-1}$) corresponded to particle densities where both larval species maintained relatively constant buccal volumes in previous experiments, displaying "normal" contractions of the buccopharyngeal musculature (Seale and Wassersug, 1979). Wet mass of most live animals was measured (after being gently blotted with a paper towel) to the nearest 0.01 g with a triple-beam balance, following the completion of each experiment. The smallest tadpole size class was weighed to the nearest mg with an analytical balance, in tared containers containing water (0.5–2.0 ml).

Experimental advantages of anuran larvae for studying the process of suspension feeding

The behavioral responses of tadpoles to particulate food (*e.g.* pumping rate adjustments) often can be observed directly (also see Seale and Wassersug, 1979). Filtering and ingestion rates can be estimated on single tadpoles, eliminating crowding effects and allowing individual variability to be assessed. Tadpole feeding responses are similar across food types, minimizing confounding effects of selective feeding (Seale and Beckvar, 1980). Individual tadpoles can cause detectable reductions in particle concentrations inside grazing chambers within a matter of minutes rather than of hours. Hence, the duration of tadpole feeding experiments can be less than one division time of the prey organism. This minimizes the confounding effect of prey growth in control and experimental chambers, a serious problem in zooplankton studies (Donaghay and Small, 1979). Data from the tadpoles (*e.g.* Seale and Beckvar, 1980; Seale *et al.*, 1982) provide a relatively clear-cut assessment of the shapes of feeding curves, compared to zooplankton data sets (*e.g.* Frost, 1975; Mullin *et al.*, 1975; Donaghay and Small, 1979), in part because the concentration ranges for tadpole feeding encompass several orders of magnitude, including those where electronic particle counters give reliable data above background.

RESULTS

Suspension-feeding dynamics

The larvae of *Rana pipiens* and *Xenopus laevis* displayed similar patterns of change in filtering rate, F, and ingestion rate, I, as functions of continously varying concentration C (Fig. 1). However, there were several species-specific differences, particularly in the behavioral mechanisms used to regulate feeding (Fig. 2). The primary response to changes in C of the *Xenopus* larva was an adjustment in pumping rate, P, and that of *Rana* was an adjustment in buccal volume, BV (Fig. 2). These general relationships are described more fully below. There was no significant difference (t test; $P > 0.05$) in the average wet mass of larvae of the two species, *R. pipiens* (1.19 ± 0.49 g; n = 7), and *X. laevis* (1.29 ± 0.26 g; n = 15). Since variables ranged over several orders of magnitude, in this paper data are plotted as log transformations for maximum resolution in lower ranges.

Using the convenient terminology of Mullin *et al.* (1975), the F peaked at an intermediate "critical" concentration (CC) and declined at both higher and lower concentrations (Fig. 1A), in both species. Below the "threshold" concentration (TC), the estimates for filtering rates were zero. The I ($=FC$) was a direct, nonlinear function of C (Fig. 1B), increasing from zero at the TC and asymptotically approaching a maximum ($I_{\max}$) at very high C. These patterns in I and F are consistent with several models previously used to describe zooplankton feeding (Mullin *et al.*, 1975; and further discussion below).

In the presence of tadpole grazing, the C eventually stabilized at a low concentration. Since the indirect method results in a positive estimate of F only with detectable changes in C through time, this stabilization resulted in an estimate of zero for F at low C (Fig. 1). Observations of zero F are plotted as $0.01\ \mathrm{ml \cdot g^{-1} \cdot h^{-1}}$ on the log-log plot in Figure 1. The concentrations where estimates of F approach zero designate "thresholds" for individual tadpoles (Fig. 1A). A tadpole placed in a yeast suspension at or below its TC caused no further reductions in yeast concentrations, but continued regular buccal pumping (Fig. 2). Hence, some yeast-laden water necessarily was being passed over the tadpole's buccopharyngeal particle-entrapping surfaces. These observations suggest that the efficiency of particle retention was reduced at low C, and that the filtering rates and buccal volumes (estimated as F/P) shown here are minimum estimates (also see Seale, 1981).

For each species, the filtering rate peaked in the vicinity of a "critical" C, the CC. The CC for *Rana* was an order of magnitude higher than that for *Xenopus* larvae (Fig. 1A). There was a great deal of variability in the C where individual tadpoles displayed their peak F. By analyzing residual plots of piecewise regressions (after Neter and Wasserman, 1974) on log-transformed data, the CC of each species was estimated (Table I). The estimated slopes of the lines describing the relationship between F and C were not significantly different for the two larval

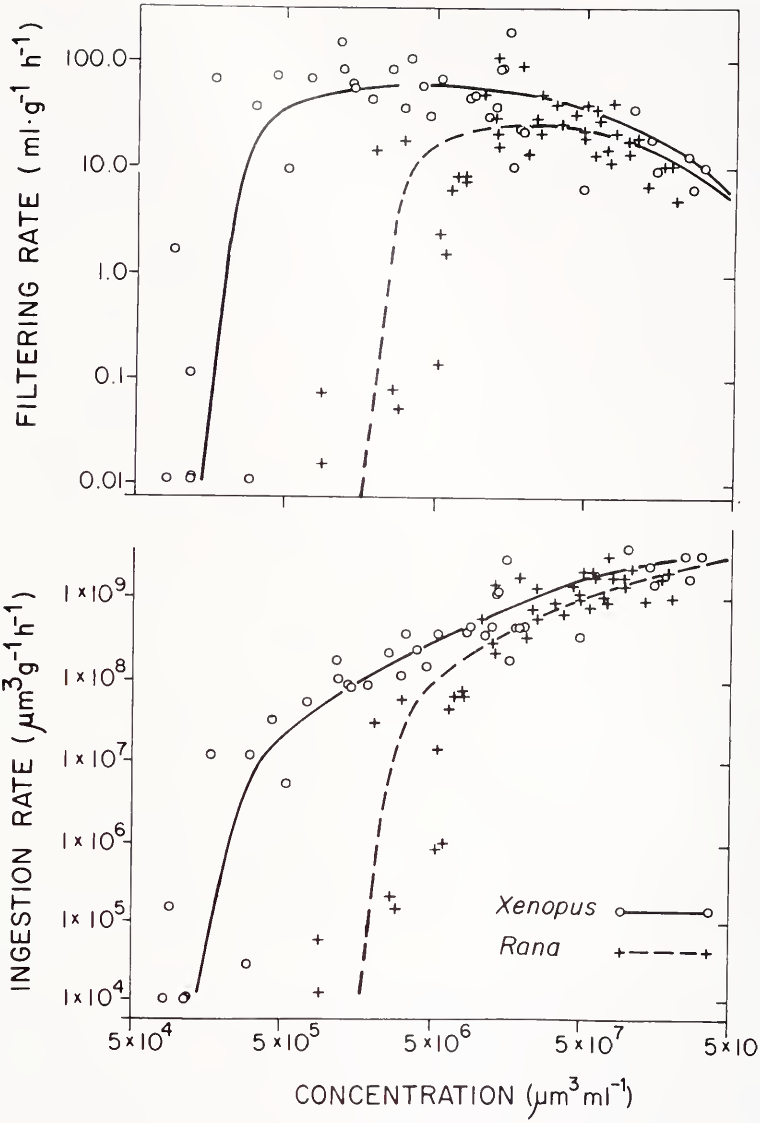

FIGURE 1. Feeding responses of *Xenopus laevis* and *Rana pipiens* larvae, as functions of continuously varying yeast concentrations. Morphologically, these two species represent extremes in feeding adaptations. Reductions in concentration through time were caused by tadpole feeding. An electronic particle counter was used to obtain indirect estimates of filtering (top) and ingestion (bottom) rates. Estimates assumed an exponential decline in yeast concentration through time and 100% particle retention. The fitted lines show the patterns of filtering and ingestion rates predicted by the Monod (Michaelis-Menten) model with a threshold: $I = I_{max} (C - TC)/[K_{1/2} + (C - TC)]$, where I is the ingestion rate, I_{max} is the maximum ingestion rate, $K_{1/2}$ is the "half-saturation constant", C is the mean yeast concentration over each sampling interval, and TC is the "threshold" concentration. Model predictions for F were derived as I/C. According to the model, ingestion rate is half-maximum at $K_{1/2} + TC$. Parameter estimates used for plots were for *Rana*: $I_{max} = 2.9 \times 10^9$ $\mu m^3 \cdot$(g wet mass) $^{-1} \cdot h^{-1}$, $K_{1/2} = 8.8 \times 10^7$ μm^3, and $TC = 1.9 \times 10^6$ μm^3; and for *X. laevis*: $I_{max} = 3.0 \times 10^9$ $\mu m^3 \cdot$(g wet mass)$^{-1} \cdot h^{-1}$, $K_{1/2} = 4.3 \times 10^7$ μm^3, and $TC = 1.9 \times 10^5$ μm^3. Of course, individual variability and experimental conditions can alter these "parameters", and other mathematical expressions for these responses can be formulated.

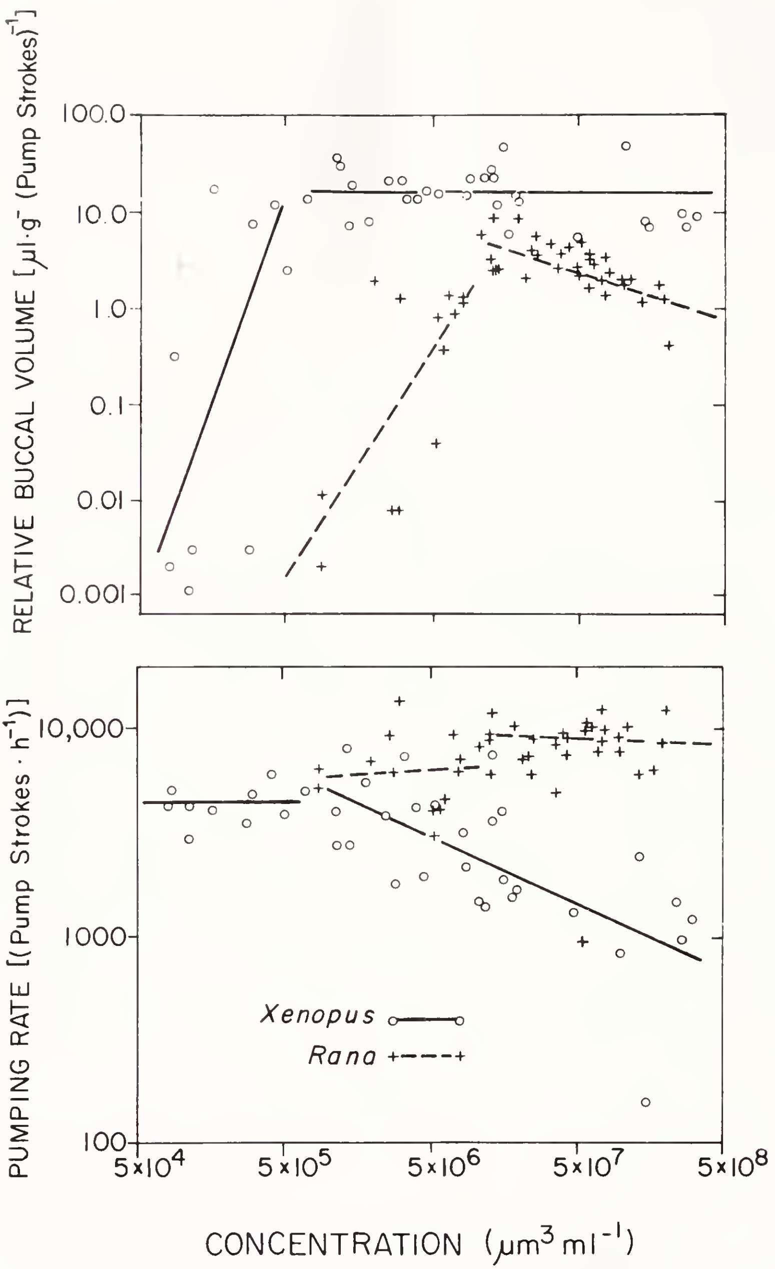

FIGURE 2. Differences in behavioral mechanisms used to regulate feeding by facultative (*Rana*) and obligate (*Xenopus*) suspension-feeding tadpoles. The major response of *X. laevis* larvae to changes in *C* was an adjustment in buccal pumping rates. *R. pipiens* larvae responded to changes in concentration primarily by altering their apparent buccal volume (volume cleared/pump stroke), apparently by adjusting contractions of buccopharyngeal muscles as well as particle entrapment efficiency. Fitted lines show piecewise linear regressions to the log-transformed data, using the "critical" concentration (Table I) as the point of discontinuity (parameter estimates in Table I).

species, either above the *CC* ($P > 0.05$), where the slopes for log *F vs* log *C* were negative for both, or below the *CC* ($P > 0.05$), where they were positive for both species (data in Table I).

The two species made these similar adjustments in *F* through different behavioral mechanisms: *Xenopus* larvae primarily regulated pumping rates, and *Rana* larvae buccal volumes (Table I; Fig. 2). With increasing *C*, the pumping rate, *P*, of the *Xenopus* larva declined exponentially from maximum at the *CC*, causing a significant negative relationship between log *P* and log *C* but not in *Rana* (Table I). Below the *CC*, neither species altered pumping rate significantly with changing *C* (Table I). For the *Rana* larva, buccal volume was a negative function of *C* above the *CC* but not for the *Xenopus* larva (Table I). Below the *CC, BV* was a positive exponential function of *C* in both species (Table I). Filtering rates declined much more rapidly than pumping rates as tadpoles reduced *C* below their *CC* (Figs. 1, 2), causing this positive *BV vs C* relationship. The rapid reductions in *BV* probably resulted from reduced particle entrapment efficiency. Filtering rates may be altered by adjusting the contractions of buccopharyngeal muscles and by changing entrapment efficiencies (Seale and Wassersug, 1979; Seale *et al.*, 1982).

In both species, *I* asymptotically approached a maximum above the *TC* (Fig. 1B). The mean of the highest observed ingestion rates, as $\mu m^3 \cdot (\text{g wet mass})^{-1} \cdot h^{-1}$, of the *R. pipiens* larvae fed on yeast ($1.59 \pm 0.30 \times 10^9$; n = 19) was not significantly different (*t* test; $P > 0.05$) than those of *R. catesbeiana* fed on algae ($1.15 \pm 0.41 \times 10^9$; n = 6) in a previous study (Seale and Beckvar, 1980). Also, the mean highest ingestion rate for yeast by the *X. laevis* larvae ($2.88 \pm 0.77 \times 10^9$; n = 7) in this study was not significantly different (*t* test; $P > 0.05$) than for algae ($3.43 \pm 1.55 \times 10^9$; n = 5) in the earlier study (Seale and Beckvar, 1980). In this study, using tadpoles well acclimated on yeast, the mean of highest ingestion rates for *Rana* and *Xenopus* larvae were not significantly different from each other (*t* test; $P > 0.05$). These maximum ingestion rates were intermediate between previous estimates for *Rana* and *Xenopus* fed on algae, where significant differences were noted (Seale and Beckvar, 1980). This suggests that the different acclimation procedures used for tadpoles of these two genera could have resulted in the species differences in maximum feeding rates observed previously (Seale and Beckvar, 1980).

Our data on the ingestion rates of *Rana* and *Xenopus* larvae *vs* concentration were fitted (after Mullin *et al.*, 1975) to a Monod (Michaelis-Menten) equation, modified to include a lower threshold for feeding (Fig. 1). All asymptotic statistics are approximate; hence, variations in the algorithm, the number of iterations, and the starting values can produce different estimates of the nonlinear parameters (Ralston and Jennrich, 1978). Also, the confidence intervals of parameter estimates based on the nonlinear algorithm are obtained by first linearizing the variables and hence are somewhat incorrect. Several solutions of the nonlinear equations for the *Xenopus* data set gave a zero estimate for *TC*: for example, $I_{max} = 3.6 \pm 1.0 \times 10^9$ $\mu m^3 \cdot (\text{g wet mass})^{-1} \cdot h^{-1}$, $K_{1/2} = 5.2\ (\pm 4.6) \times 10^7$ μm^3, and $TC = 0.0\ (\pm 4.5) \times 10^6$ μm^3. This result apparently was a statistical artifact: variability in individual feeding "thresholds" resulted in low statistical repeatability. Zero filtering rates were, in fact, measured for several individual tadpoles at positive, measurable yeast concentrations (Fig. 1). None of the nonlinear solutions for the *Rana* larvae gave zero estimates for *TC*. The fitted line (Fig. 1) shows the solution giving the smallest estimated threshold for *R. pipiens* tadpoles. Additional solutions with "well-behaved" residual plots included $I_{max} = 2.9\ (\pm 1.3) \times 10^9$ $\mu m^3 \cdot (\text{g wet mass})^{-1} \cdot h^{-1}$, $K_{1/2} = 2.7\ (\pm 3.7) \times 10^8$ μm^3, and $TC = 3.3\ (\pm 5.4) \times 10^6$ μm^3, for *R. pipiens*.

TABLE I

Estimated parameters for relationships between feeding responses and concentration in anuran larvae (Rana *and* Xenopus).

	Below "critical" concentration	Above "critical" concentration
Filtering Rate		
Rana pipiens	$\log FR = -14.63\ (\pm 11.55) + 2.23\ (\pm 1.76)^{*} \log C$	$\log FR = 5.17\ (\pm 1.82) - 0.50\ (\pm 0.24)^{***} \log C$
Xenopus laevis	$\log FR = -18.78\ (\pm 24.93) + 3.52\ (\pm 4.72) \log C$	$\log FR = 4.20\ (\pm 0.93) - 0.37\ (\pm 0.13)^{***} \log C$
Pumping Rate		
Rana pipiens	$\log PR = 2.73\ (\pm 2.58) + 0.16\ (\pm 0.39) \log C$	$\log PR = 4.62\ (\pm 1.53) - 0.10\ (\pm 0.20) \log C$
Xenopus laevis	$\log PR = 3.91\ (\pm 3.11) - 0.07\ (\pm 0.59) \log C$	$\log PR = 5.09\ (\pm 1.12) - 0.27\ (\pm 0.16)^{**} \log C$
Buccal Volume		
Rana pipiens	$\log BV = -16.22\ (\pm 10.84) + 2.35\ (\pm 1.65)^{**} \log C$	$\log BV = 4.40\ (\pm 1.54) - 0.52\ (\pm 0.20)^{***} \log C$
Xenopus laevis	$\log BV = -19.08\ (\pm 24.01) + 3.46\ (\pm 8.44) \log C$	$\log BV = 1.57\ (\pm 0.96) - 0.05\ (\pm 0.14) \log C$

H_0: $\beta = 0$
* $0.05 > P > 0.01$
** $0.01 > P > 0.001$
*** $0.001 > P \geq 0.0001$

Estimates of parameters (±95% confidence intervals), from linear regressions on log-transformed data, of several variables related to feeding regulation in obligate (*Xenopus*) *vs* facultative (*Rana*) suspension-feeding tadpoles. Data in Fig. 1, 2. Parameters estimated from data collected on tadpoles feeding on yeast concentrations above and below the "critical" concentration for each species. Points of inflection on piecewise regressions determined the "critical" concentrations: *R. pipiens* $7.9 \times 10^6\ \mu m^3 \cdot ml^{-1}$; and *X. laevis* $6.3 \times 10^5\ \mu m^3 \cdot ml^{-1}$.

Buccal volume as a function of individual size

The apparent buccal volume (*BV*: volume cleared/pump stroke) for *Xenopus* larvae was larger than that for comparably sized *Rana* larvae (Fig. 2). An analysis of covariance, using individual snout–vent length (*SV*) as the covariate, indicated that the *Xenopus* larvae had larger apparent buccal volumes than did the *Rana* larvae, over all size classes examined ($F = 148.5$; $P = 0.0001$). A double-logarithmic transformation of the data indicates that log *SV vs* log *BV* are linearly related (Fig. 3). Therefore, the relationship between *SV* and *BV* could be fitted to a power function for cohorts of both *X. laevis* and *R. sphenocephala*. Using nonlinear regression (after Zar, 1968), the parameters in the power function were: *Xenopus* $BV = 7.63\ (\pm 4.03)\ SV^{1.86\ (\pm 0.74)}$; *Rana* $BV = 1.23\ (\pm 0.65)\ SV^{2.38\ (\pm 0.86)}$. The exponents of the two power functions were not significantly different from each other or from 2 ($P > 0.05$). The estimates of the exponents for both species was less than 3, indicating negative allometry (Huxley, 1932, 1950).

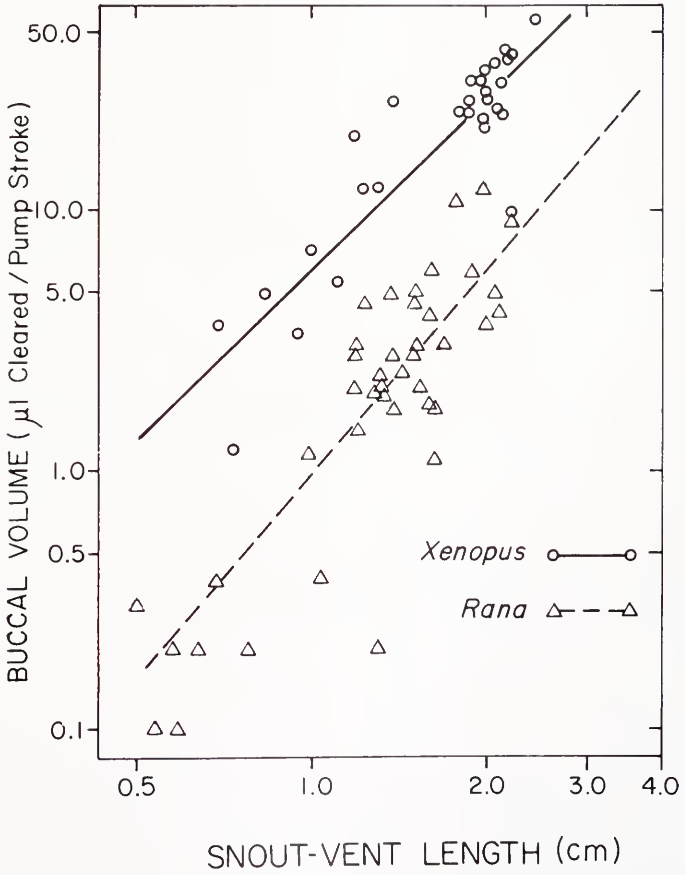

FIGURE 3. Apparent buccal volumes (*BV*) of *X. laevis* and *R. sphenocophala* larvae, as functions of individual sizes, measured by snout–vent lengths (*SV*). This double-logarithmic transformation of the data indicates that the relationships can be described by power function ($BV = aSV^{b}$), with $b < 3$, suggesting negative allometry.

Discussion

Adaptations of obligate and facultative suspension-feeding tadpoles

Morphological differences between *Xenopus* and *Rana* larvae reflect their obligate and facultative suspension-feeding habits. The *Xenopus laevis* larva is an obligate, midwater suspension feeder, and lacks the keratinized beak, dual water pump, and pharyngeal bypass of the facultative suspension-feeding *Rana* larva (Gradwell, 1971, 1972a, b, 1975; Wassersug and Hoff, 1979; Wassersug, 1980). A keratinized beak enables the *Rana* larva to produce suspensions from attached particles, which then can be ingested. In addition, the *Rana* larva's potential feeding versatility is demonstrated by buccopharyngeal structures which allow great flexibility in adjusting buccal volume (Wassersug, 1980). By altering contractions of the musculature associated with the buccal pump, *Rana* tadpoles can regulate buccal volume over as much as 3 orders of magnitude to adjust to changing particle concentrations (Fig. 2; Seale and Wassersug, 1979; Wassersug and Hoff, 1979).

The *Xenopus* larva has an unusually large buccal volume, large branchial baskets with dense gill filters, and extensive branchial food traps (Wassersug and Hoff, 1979; Wassersug and Rosenberg, 1979; Wassersug, 1980; Seale *et al.*, 1982). Compared to *Rana*, a *Xenopus* larva has a relatively short lever arm on the cartilagenous ceratohyal which delivers the driving force of the tadpole's buccal pump (Wassersug and Hoff, 1979). Hence, a relatively shallow contraction of the buccal pump musculature can cause a disproportionately large volume of water to be displaced in *Xenopus* (Wassersug and Hoff, 1979). This means the *Xenopus* larva can clear a larger volume of water than *Rana*, with each pump stroke. However, the arrangement of cartilage and musculature in the *Xenopus* (contra the *Rana*) tadpole limits its ability to adjust the amplitude of the pump stroke (Gradwell, 1971, 1972a, b; Wassersug and Hoff, 1979; Wassersug, 1980).

Buccal volumes in growing Rana *and* Xenopus *larvae*

Prior to the onset of metamorphosis, tadpole body proportions remain relatively constant, indicating isometric growth (Wassersug, 1975). However, a closer examination suggests some subtle trends away from isometry in certain body shape parameters. For example, from a geometric model of the tadpole's buccal pump, Wassersug and Hoff (1979) found that buccal volume scales as a power function of snout–vent length, with the exponent less than three, *i.e.* negative allometry. They, however, reached this result from an examination of the morphology of preserved specimens. My results (Fig. 3) with living *R. sphenocephala* and *X. laevis* larvae confirm this relationship.

Suspension-feeding dynamics of Rana vs Xenopus *larvae*

The qualitative shapes of curves describing relationships between filtering and ingestion rates *vs* particle concentration, for *Xenopus* and *Rana* larvae, were similar in three major respects (Fig. 1): 1) with increasing particle densities, ingestion rate asymptotically approached a maximum; 2) the filtering rate peaked at an intermediate "critical" concentration; and 3) estimated filtering and ingestion rates declined rapidly from maximum at the "critical" concentration to zero at a lower "threshold" concentration. These feeding curves confirmed previous results, which applied somewhat different methods (Seale and Wassersug, 1979; Seale and Beckvar, 1980; Seale *et al.*, 1982). Hence, these patterns of feeding regulation (Fig. 1) apparently are characteristics of the animals, rather than experimental artifacts. Furthermore, these patterns were consistent with mathematical equations previously developed to describe zooplankton feeding (further discussion below).

Unlike earlier studies with algae (Seale and Beckvar, 1980), the larvae of *Rana* and *Xenopus* had approximately the same maximum ingestion rates, when fed yeast, adjusted for tadpole mass (Fig. 1). As previously observed (see Seale and Wassersug, 1979), the concentration "threshold" for the *Xenopus* larva was lower than for *Rana*. However, the "threshold" observed for *Xenopus* in this study was lower than that previously obtained (Seale *et al.*, 1982), and was not significantly different from zero (Fig. 1, and associated text). The reasons for these quantitative differences are not clear, but may be related to differences in food quality and methodology.

The precise mechanisms used to adjust filtering and ingestion rates differed in the two species. The larva of *X. laevis* altered filtering rates almost exclusively by varying pumping rate, and the *R. pipiens* larva by adjusting buccal volume (Fig. 2). These observations were consistent with morphological adaptations for feeding (described above), and with preliminary studies (see Seale and Wassersug, 1979; Wassersug and Hoff, 1979; Seale *et al.*, 1982). The *Xenopus* larvae maintained near-maximum filtering rates over a much wider concentration range than *Rana* (Fig. 1), primarily by clearing a larger volume of water with each pump stroke (Fig. 2). Over all size classes, *Xenopus* larvae had larger buccal volumes than comparably-sized *Rana* (Fig. 3).

The obligate suspension-feeding larva of *Xenopus* can survive solely on suspended particles over a wider range of concentrations, but the facultative suspension-feeding *Rana* larva has greater behavioral plasticity in its feeding. *Xenopus* larvae can remove particles at very low concentrations, within the range of those in most oligotrophic lakes (Kalff and Knoechel, 1978). *Xenopus* tadpoles have been observed in eutrophic African ponds (R. Inger, pers. comm.), but energetically they are capable of surviving in more oligotrophic waters (Seale *et al.*, 1982). *Rana* larvae can switch their feeding to fit available food (Seale, 1980). *Rana* tadpoles ingest epiphytic (Dickman, 1968), epibenthic (Calef, 1973), as well as suspended (Seale and Wassersug, 1979; Seale and Beckvar, 1980) microscopic organisms and detritus.

Tadpole feeding compared to predictions of general suspension-feeding models

Models are valuable only if they can be used as tools for increasing our understanding of important ecological questions, for example: How effectively does the model describe the observed shapes of feeding curves? Are the mechanisms used by the animal to regulate its feeding consistent with the model's assumptions?

Two classes of models describe the general shapes of the feeding curves exhibited by the tadpoles (Fig. 1 and description above): 1) "curvilinear" models, modified to include a lower concentration "threshold" below which feeding ceases; examples are models which assume a rectangular hyperbola (Michaelis-Menten or Monod) or a negative exponential (Ivlev) equation, both of which predict that feeding will approach a maximum asymptotically at high concentrations (see Mullin *et al.*, 1975); and 2) models which assume suspension feeders adjust ingestion to maximize energy return at each food concentration (Lam and Frost, 1976; Lehman, 1976). Tadpoles regulate their feeding by complex, species-specific, behavioral adjustments (Fig. 2), which probably can be understood best in terms of energy requirements. The shapes of the feeding curves relating I and F to C compare extremely well with predictions for the simple modified Monod model (Fig. 1), and fairly well with the more complex energy optimization models. The major difference is that the energy optimization models do not predict an absolute feeding "threshold," such as that observed in tadpoles (Fig. 1). Instead they predict a reduction in filtering and ingestion to low, positive rates, as concentrations decline (Lehman, 1976). Doyle (1979) has demonstrated that energy optimization models are mathematically compatible with the simple Monod model. Both may be derived from an assumption of regulation by gut satiation: this could be a fundamental regulatory mechanism for suspension feeding, leading to similar feeding patterns in phylogenetically diverse animals.

Tadpoles can make behavioral adjustments to compensate for reductions in particulate food: 1) pumping rates (hence energy expenditures) decline in animals after at least 1 h of being acclimated to very low particle levels (Seale *et al.*, 1982); and 2) animals may move to higher particle concentrations. Seale *et al.* (1982) suggested that as particle levels decline to a C near the observed "threshold," the energy expended by a tadpole in filtering suspensions probably would not be balanced by energy gains, providing an impetus for a behavioral shift in feeding regime (also see below).

The modified Monod model can be derived from several biologically reasonable assumptions concerning mechanisms for feeding regulation: 1) Feeding surfaces can become "saturated." The asymptote in the feeding curve (maximum ingestion rate) corresponds to highest concentrations, where filtering surfaces become filled (Parker, 1975; Real, 1977). 2) Ingestion rate is governed by prey densities and predator search time (related to encounter probability) and handling time (after Holling, 1959, 1965; Crowley, 1973). 3) Feeding can be governed by gut-filling rate or satiation (Doyle, 1979). Although none of these mechanisms have been shown conclusively to operate for tadpoles, they are consistent with what is known about tadpole feeding (see Seale and Wassersug, 1979; Seale *et al.*, 1982). For example, tadpole filters show no signs of particle accumulations except at very high concentrations (Seale *et al.*, 1982), consistent with assumption 1.

The rectangular hyperbola is mathematically isomorphic with Holling's (1959, 1965) "invertebrate predator" or "Type II" functional response (Parker, 1975; Real, 1977). The "Type III" or "vertebrate predator" model, a sigmoidal feeding function, is similar in shape to feeding curves predicted by energy-optimization models (Lehman, 1976), and represents the effects of learning and of alternative prey on animal feeding, shifting from the "Type II" curve (Real, 1977). Tadpole feeding cannot be described accurately with the "rectilinear" model of Rigler (1961), the algebraic analog of Holling's "Type I" or "suspension feeder" model, because: 1) tadpole filtering surfaces are not saturated at the "critical" concentration (Seale *et al.*, 1982); and 2) tadpole filtering rates decline below the "critical" concentration, rather than remaining constant as Rigler assumed. Obviously, Holling's (1959, 1965) phylogenetic and functional categories for these models are restrictive, although the models themselves are useful for describing general feeding responses.

The "threshold" in the modified Monod model could represent either a discontinuity in feeding (as in Fig. 1), or a behavioral response to environmental heterogeneity. The data indicate that individual tadpoles can reduce feeding rates to zero at low concentrations (Fig. 1), but these indirect feeding data cannot determine if this "threshold" represents an actual reduction in filtering rate, a "compensation point" where ingestion is balanced by particle release, or a reduction in entrapment efficiency (Seale, 1981). Theoretically, "threshold" feeding behavior also can represent the case where a predator becomes inactive or less efficient at low concentrations. In addition, the threshold in the modified Monod model provides a simple first approximation to a mathematical expression of the potential effects of a facultative suspension feeder on planktonic organisms, *e.g.* the ability of the *Rana* tadpole to switch feeding from the water column to the sediments or other alternative food sources at low particle concentrations (Seale, 1980). This equation could also be used as a simple model of other discontinuities in feeding, such as migration to a more concentrated patch in a heterogeneous environment, or a temporary inability to ingest prey in a spatial refuge which can become available for predation (*e.g.* algae attached to substrates may later become suspended).

Although the modified Monod model describes tadpole feeding data fairly well (Fig. 1), "parameters" in the model are somewhat sensitive to individual variability, past history, and environmental conditioning (also see Donaghay and Small, 1979). In these studies, non-zero "thresholds" were observed on individual larvae (Fig. 1). However, the "threshold" C varied from tadpole to tadpole. This individual variability probably was a factor causing the population parameter estimate for the threshold to be zero for some nonlinear regressions. Measurements made on many animals at once (as in zooplankton feeding studies: Mullin *et al.*, 1975) should not be expected to provide unambiguous parameter estimates for a "threshold," even if one exists.

Individual variability aside, "parameter" estimates for feeding models cannot be interpreted as physiological constants for the species. For example, the pumping rates of tadpoles acclimated 1 h to one C were lower than for other tadpoles experiencing that same C while causing continuous reductions in particle concentrations (Seale *et al.*, 1982). In continuous reduction experiments, tadpoles maintain maximum pumping rates at very low C (Seale *et al.*, 1982; Fig. 2, this study), which probably causes the apparent $K_{1/2}$ to be somewhat lower than would be seen in well-acclimated tadpoles. F. M. Williams (unpubl.) has predicted such a distortion in the feeding curve when feeding effort remains disproportionately high at relatively low concentrations, and has named this the "Avis Effect" ("We try harder"). Using a flow-through system, Seale (unpubl.) also has shown that tadpoles exhibit higher pumping rates at given C after having been maintained for some time in low C, compared to rates observed after tadpoles were held in higher C. Feder (1981) demonstrated that for *Xenopus* larvae a number of factors, including time of day and stress from physical disturbances, can cause dramatic changes in respiratory activities, which are linked to feeding in tadpoles. Nevertheless, when measured under similar experimental conditions, differences in feeding parameters may be useful in comparing relative feeding capabilities, as for evaluating potential competitive interactions among different species and sizes of tadpoles (see also Seale, 1980, 1981; Seale and Beckvar, 1980).

Evolutionary considerations and the impact of vertebrate suspension feeders on plankton

A *Xenopus* larva can clear a much larger volume of water with each pulsatile contraction of its buccopharyngeal musculature than can a *Rana* larva. Hence, the *Rana* larva must pump more rapidly to obtain the same amount of food at any given concentration (Figs. 1, 2). Because of its slower pumping rate (Fig. 1), the energy expended by the *Xenopus* larva probably is less than that needed by the *Rana* larva to obtain a comparable amount of food at any given particle concentration.

These deductions suggest a partial explanation for the differences in feeding regulation in *Xenopus* compared to *Rana* larvae, assuming such differences reflect adaptations to the environment. Because of low seasonal variability, primary production tends to remain relatively high and constant through time in South African, compared to temperate, waters (Greenwood, 1976). The *Xenopus* larva is endemic to South Africa. Over evolutionary time, an environment with a dependable source of suspended particles should justify the extra energetic costs needed to maintain this larva's elaborate feeding structures (see also Wassersug, 1980; Seale *et al.*, 1982). No anurans with obligate suspension-feeding larvae are endemic to temperate North America. The same morphological specializations which enable a *Xenopus* larva to maximize the volume cleared per pump stroke lead to inflexibility in the intensity of the pump stroke (Wassersug and Hoff, 1979; Wassersug, 1980). Extreme variability and unpredictability of particulate densities characterize the temperate environments inhabited by facultative suspension-feeding anuran larvae, such as *Rana* tadpoles. The ability to adjust pump stroke intensity allows them to adjust the energy costs associated with suspension feeding. In addition, their greater flexibility in feeding behavior improves their ability to adapt to a more unpredictable environment (additional discussions above and in Wassersug, 1975, 1980; Seale, 1980). Tadpoles overwintering in temperate habitats encounter low primary production rates but can exploit alternative foods (Seale, 1980). The *Rana* larva's ability to shift grazing may be related, at least in part, to predator avoidance. In the presence of predation, the *Rana* larva can feed on alternative foods, whereas the *Xenopus* larva is constrained to remain in the water column. This may imply a fundamental difference in adaptations to avoid predation in these two species. In support of this hypothesis, the *Xenopus* larva is transparent whereas North temperate tadpoles, such as *Rana*, tend to be colored cryptically, *e.g.* to blend in with the sediments.

One may speculate on the probable trophic dynamics in ecosystems dominated by large vertebrate suspension feeders. All such animals, including tadpoles, are several orders of magnitude larger than their planktonic prey. For example, an average *Xenopus* larva weighs 10^4–10^5 times as much as an average copepod, but can ingest prey of the same size taken by copepods, or smaller. Since these vertebrates feed on lower trophic levels than most, the number of links in the food chain is reduced, potentially leading to high trophic efficiencies. Circumstantial evidence for unusually efficient transfer of energy from autotrophs to vertebrates have been noted in ecosystems dominated by tadpoles (Seale, 1980) and by planktivorous fishes (Hecky and Fee, 1981; Hecky and Kling, 1981). In both ecosystems, algal standing crops were about an order of magnitude lower than expected to support these vertebrate populations, if trophic efficiencies were "normal." At times vertebrate biomass exceeded phytoplankton biomass (Seale, 1980; Hecky and Fee, 1981). Assuming gut contents comprise 30–50% of a tadpole's biomass (after Calef, 1973; Feder, 1981), during these trophic "inversions" the algal biomass inside tadpoles at times equalled or exceeded that in the water column (Seale, 1980). At maximum tadpole densities, the estimated rates of material flux through the *Rana* larvae exceeded the total (per pond) rates of primary production (Seale, 1980). These field observations cannot distinguish between high trophic efficiencies and additional sources of energy for the vertebrates, such as chemoautotrophic and heterotrophic production by bacteria (Hecky and Kling, 1981). The tadpoles released large amounts of dissolved organic matter, which could have promoted bacterial growth and/or inhibited algal growth (Seale, 1980).

A number of adult or larval aquatic vertebrates can entrap plankton several orders of magnitude smaller than their own body mass. Examples are larval lampreys (Moore and Mallatt, 1980), filter-feeding fishes (Durbin and Durbin, 1975), baleen whales (Brodie, 1975), and tadpoles. Some patterns of behavior exhibited by these animals represent adaptations to variability in rates of supply of particulate food, among other factors. Seasonal variability in levels of such particles may have been an important evolutionary impetus for the development of metamorphosis in anurans (Wassersug, 1975). An intriguing analogy may be drawn between metamorphosis in vertebrates with suspension-feeding larvae, such as anurans and lampreys, and migratory behavior in other suspension-feeding vertebrates, such as whales and fish. Some cetaceans, such as the fin whale, filter huge quantities of seasonally available krill from Antarctic waters during a few months each year; blubber stored during these summer excursions then supplies energy for migrations into warmer waters, where food is limited but maintenance energy requirements are minimal (Brodie, 1975). Adult mullets are open-water oceanic fish which lay their eggs at sea. The hatchlings then migrate into estuaries where they feed on suspended plankton in this highly productive habitat for about a year (Bardach *et al.*, 1972). Lamprey have suspension-feeding larvae which take advantage of seasonally abundant suspended particles in freshwater streams, but as adults are parasitic on fish (Moore and Mallatt, 1980).

The first vertebrates almost certainly were suspension feeders (Romer, 1970). Divergence from this feeding mode probably was stimulated, in part, by the vertebrates' comparatively slow population responses to variations in particulate food. In contrast, zooplankton populations may respond within hours to variations in particulate food, for example, by changing egg production, hatching success, developmental time, adult size, reproductive mode (sexual *vs* asexual), and life span (Boraas, 1980). A number of behavioral adjustments, such as migration, metamorphosis, threshold feeding, and prey switching, may serve to minimize overexploitation of the prey of vertebrate suspension feeders, while insuring adequate food to supply energetic needs.

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L3

GENETIC AND NON-GENETIC VARIABILITY IN TEMPERATURE TOLERANCE OF THE COPEPOD *EURYTEMORA AFFINIS* IN FIVE TEMPERATURE REGIMES

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ABSTRACT

Genetic and nongenetic variation in temperature tolerance was measured in populations of copepods grown in two environments varying between 10° and 23°C on 26 day (SW) and 26 week (LW) cycles and in three constant environments at 10°C, 15°C, and 23°C.

Genetic variation was maintained and expressed in both sexes in the cycling environments, but declined in males in the 23°C constant environment, perhaps indicating constant directional selection.

Physiological variation was similar in males and females, in contrast to earlier results, again suggesting an effect of selection. There was evidence of selection for physiological flexibility in males in the 23°C and 15°C environments.

A question arising was why sexual dimorphism in genetic variation and in physiological variation was maintained in nature but reduced, reversed, or even eliminated in the laboratory environment. Random drift was not a plausible explanation.

INTRODUCTION

The copepod *Eurytemora affinis* is a seasonally dominant species in the zooplankton of Chesapeake Bay. The greatest densities occur in winter and early spring. Although water temperatures can reach 30°C in summer and 0°C in winter, no resting stages have been found in this species.

Since generation time in *Eurytemora* varies from about 10 days at 23°C to 2 months at 4°C, breeding individuals do not experience the entire 30°C range in temperature. Thus, the species may adapt to temperature variation either partly through changes in gene frequency, progeny being adapted to the temperature at which their parents were selected, or entirely physiologically. Some physiological adjustment is inevitable since individuals experience some variation in ambient temperature, in space if not in time.

Previous workers, such as Marshall and Jain (1968), Levins (1969), and Selander and Kaufman (1973), have suggested that in populations or species with relatively large amounts of physiological variation there is less genetic variation, and *vice versa*. In the case of *Eurytemora* the dimorphism is between the sexes, females having higher physiological and lower expressed genetic variation than males (Bradley, 1978a, b). Obviously in a species such as *Eurytemora* with obligate

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Abbreviations: LW, long wave temperature cycling (1° per week); SW, short wave temperature cycling (1° per day).

sexual reproduction, the underlying genetic variation must be the same in the two sexes, whether or not that variation is expressed.

The genetic and physiological variation observed in the two sexes can be considered as potentials for adaptation. Thus, females apparently have the greater capacity for internal adjustment to temperature change and males are more likely to be selected according to their genotypes. Such selection has been demonstrated using laboratory environments (Ketzner and Bradley, 1982). Populations maintained in the five different temperature environments, to be described later in this paper, diverged genetically. Genetic differences were also demonstrated between progeny of animals collected from intake and discharge waters of steam electric power plants (Bradley, 1978a; LaBelle and Bradley, 1982). However, variation in temperature, even over a wide range, is not sufficient by itself to produce genetic change. No genetic differences, but physiological differences were found between populations collected at different seasons in the wild (Bradley, 1982). Whether the genetic or physiological potential is the more important seems to depend on the rate rather than on the magnitude of the variation in temperature (Ketzner and Bradley, 1982).

When natural selection occurs, as it did in the five temperature environments just mentioned, we expect a reduction in genetic variation. We also expect a reduction in total phenotypic variation, of which the genetic variation is a part. Furthermore, to the extent that the potential for physiological change is important in successful reproduction, and assuming this potential varies genetically, we may find a reduction in the genetic variation in physiological potential or, in this case, in the ability to acclimate to higher temperatures.

The present paper examines phenotypic, genetic and non-genetic (largely physiological) variation in temperature tolerance of populations of *Eurytemora affinis* kept in five temperature environments for three years. Non-genetic variation is also examined as the response in temperature tolerance of individuals exposed to 23°C for 24 h. Genetic variation in this non-genetic response is also examined in the different environments.

Materials and Methods

The copepods used to stock the cultures initially were collected from Bear Creek, east of Baltimore Harbor in the Upper Chesapeake Bay, Maryland.

Environments and sampling

The temperature environments imposed on the various cultures were: constant at 10°C (10°C), constant at 15°C (15°C), constant at 23°C (23°C), cycling at 1° per day between 10 and 23°C (Short Wave or SW), and cycling at 1° per week between 10 and 23°C (Long Wave or LW).

The 23°C temperature was at or near the limit at which cultures could be maintained; the 10°C temperature was not the lowest possible temperature but allowed sufficient turnover of generations for selection to take place. Between 10°C and 4°C the generation time in *Eurytemora* increases from about one month to two months. The 15°C temperature, with generation time around three weeks, served as a control. Generation time is around 10 days at 23°C. The SW environment passed through the range of temperatures in as little as 13 days; thus, given the generation times at the various temperatures, most individuals were exposed to all temperatures. The LW environment, on the other hand, changed sufficiently slowly, taking at least 13 weeks to cover the range, that individuals were exposed to only a fraction of the temperatures between 10°C and 23°C.

Each environment had approximately 40 ovigerous females initially. Samples of animals were withdrawn for testing directly or for raising progeny. At the time of sampling, the cycling environments were passing through 15°C, and the temperature was increasing. Progeny were raised at a constant (15°C) temperature, to isolate genetic differences among cultures, maternal influences on temperature tolerance having been shown to be minimal (Bradley, 1978a).

The assay for temperature tolerance

The assay for temperature tolerance was the same as used in Bradley (1978a). Temperature tolerance was based on the time, in minutes, until animals entered a coma after they were abruptly placed in 32°C water which was increased by 0.5°C every 5 min thereafter. Individual animals were placed in 2 ml of water in vials partially immersed, in racks, in an aquarium filled with water and heated by a thermostatically controlled heating-stirring unit. The assay was done on 12 animals at a time and usually took 30 min or less to complete, depending on when the last animal became comatose. Vials containing comatose animals were removed and the animals allowed to recover. This assay was shown to be an accurate predictor of survival at high temperatures, with the obvious advantages of being short-term and non-lethal (Bradley, 1976).

Temperature tolerance was measured on each animal before and after acclimation for 24 h at 23°C. Males and females were assayed separately, 12 in each run.

Measurement of genetic and non-genetic variation

Differences observed between copepods in temperature tolerance, or between individual organisms in any quantitative trait, may be the result of their different phenotypes or of their external or internal environments. Thus, the totality of differences or the variation among individuals, usually summarized statistically as the variance, can be partitioned into genetic and non-genetic variation. These two components may be further divided, giving four components of variance as follows:

$$\sigma_P{}^2 = \sigma_A{}^2 + \sigma_{NA}{}^2 + \sigma_{E_g}{}^2 + \sigma_{E_s}{}^2$$

where $\sigma_P{}^2$ = phenotypic variance, $\sigma_A{}^2$ = additive genetic variance (due to the sum of the individual gene effects), $\sigma_{NA}{}^2$ = genetic variance due to interaction between genes in pairs (dominance) or between pairs of genes (epistasis), $\sigma_{E_g}{}^2$ = the variance due to changes in the external environment, and $\sigma_{E_S}{}^2$ = the variance due to internal (physiological) changes in individuals. Obviously, the first two components together are the genetic variation and the latter two the non-genetic or environmental variation. The components of particular interest in this study were the $\sigma_A{}^2$ and $\sigma_{E_S}{}^2$ terms, representing, respectively, the potential for genetic change, since only individual genes and not parental gene combinations are transmitted, and the potential for physiological change.

Estimates of $\sigma_A{}^2$, the additive genetic variance, referred to also as genic variance, were obtained from the variance among broods, estimated from analysis of variance. Since one-half the additive genetic variance in a population is among broods and the other half within broods, the variance among broods is an estimate of one-half of the additive genetic variance.

Using full-sibs instead of half-sibs to estimate genic or additive (as opposed to total genetic) variance seemed justifiable since there is little sperm storage and no maternal and non-additive genetic variation was observed in temperature tolerance in *Eurytemora* (Bradley, 1978a). Both of these components of variation would contribute to the variance among broods and thus inflate the estimates of additive genetic variance. The sampling variance (and hence the standard deviation) of each estimate was derived from the analysis of variance by the method described in Brownlee (1965). Further details of the methods of analyses appear in Bradley (1978a).

Physiological variation, the other component of variation in temperature tolerance which was of particular interest in this study, was measured in two distinct ways. One estimate was obtained by subtracting the estimate of additive genetic variance from its corresponding phenotypic variance. The estimate of ${\sigma_{E_S}}^2$ was thus: ${\sigma_P}^2 - {\sigma_A}^2$. The justification for this estimate was that non-additive genetic variance (${\sigma_{NA}}^2$) and variance due to general environmental effects (${\sigma_{E_g}}^2$), at least in a constant environment, were both found to be negligible, as was stated earlier.

The second estimate of physiological variation was obtained by exposing individuals to a high temperature (24°C) for 24 h, measuring temperature tolerance before and after the exposure. Previous work (Bradley, 1978b) had shown that the 24°C temperature was sufficient to induce dramatic shifts in temperature tolerance but without mortality. Although acclimation was not complete at 24 h there was little interaction (in particular change in ranking of individuals) as animals changed in tolerance over the next 24 h or 48 h period. Thus, the short-term change could be assumed to represent the relative potentials of individuals for physiological or individual adjustment.

Since tolerances were measured before and after the acclimation just described, two further sets of measurements were possible. First, additive genetic variance in temperature tolerance after acclimation could be estimated, just as it was measured before acclimation. Second, the additive genetic variance in the degree to which individuals acclimated could also be measured. We realize that this variance is a function of the variances in temperature tolerance before and after acclimation and the covariance between them.

Additional data on fitness traits

Other data were collected on individuals and broods sampled from the environments. Egg production in a female was estimated by isolating an ovigerous female in a droplet of water and counting the eggs in her egg sac. Sex ratio was measured as the ratio of males to the total number of adults in each brood, viability as the number of adults expressed as a fraction of the number of eggs.

Finally, in order to relate temperature tolerance and other fitness traits by brood, data were collected on egg production, viability and sex ratio as well as on temperature tolerances of male and female progeny, the latter to measure average tolerances in each brood. These animals were wild collected, not derived from the five environments.

RESULTS

Data on genetic and non-genetic variation on temperature tolerances of progeny from the five temperature environments were collected over a six month period after one year exposure of cultures and again after three years. The data from the two collection periods and from the two sexes of progeny are reported separately in each of the tables.

The phenotypic variances in temperature tolerance shown in Table I suggest effects of acclimation, sex and culture environment. In the first set of data (after 1–1½ years) females were more variable than males, but after three years the variances were quite comparable. In the first set of data both sexes increased in variance after acclimation by comparable amounts, but in the later tests, after three years, female variances increased much less than did male variances following acclimation, and in some cases actually decreased.

Comparing phenotypic variances among the environments did not reveal any evidence of selection, with the possible exception of the lower variance of male progeny from the 23°C environment after three years. Note that the 10°C and 23°C variances in male progeny changed in opposite directions, before and after acclimation, between the first and second measurements.

The additive genetic variances in male progeny listed in Table II provide evidence for selection. They were lower in the SW and LW environments in the earlier data and in the 23°C, SW and LW environments in the later data. In these same environments the male variances were less than the corresponding female variances, suggesting that the genetic consequences of selection were different in males and females. The expression of additive genetic variance generally increased following acclimation, by about 100% in the first set of measurements and by about 30%, on average, in the second.

Estimates of physiological variance are shown in Table III, obtained by subtracting the additive genetic variances from their corresponding phenotypic vari-

TABLE I

Phenotypic variances in temperature tolerance before and after acclimation in male and female progeny from five environments on two occasions.

Environment	Preacc.	Postacclimation	Preacc.	Postacclimation
After 1–1½ years	Male		Female	
I 10°C	18.0	31.5	26.5	33.9
23°C	25.4	41.9	25.4	34.3
II SW	21.1	35.7	29.3	46.6
15°C	24.5	42.8	25.4	53.2
III LW	14.4	33.5	16.9	44.3
15°C	13.2	43.5	13.9	37.5
			(240 animals per estimate)	
After 3 years				
10°C	33.1	30.8	28.8	50.5
15°C	20.8	30.2	23.5	16.5
23°C	14.0	26.3	26.8	25.6
SW	21.7	41.0	24.3	37.3
LW	18.0	50.4	21.7	25.1
			(80 animals per estimate)	

The 10°C, 15°C and 23°C were constant environments; the SW and LW cycled at 1°/day and 1°/week between 10° and 23°C. The first experiment was done with three pairings of environments over six months.

TABLE II

Estimates, with standard errors, of additive genetic variance in temperature tolerance before and after acclimation in male and female progeny from five environments.

Envir.	Preacc.	Postacclimation	Preacc.	Postacclimation
After 1–1½ years	Male		Female	
I 10°C	15.1 (1.3)	31.5 (3.6)	13.5 (2.1)	22.7 (3.0)
23°C	15.5 (1.4)	10.9 (2.0)	14.2 (2.2)	16.8 (2.3)
II SW	5.7 (1.1)	16.8 (2.0)	20.5 (2.2)	45.7 (4.2)
15°C	21.6 (2.0)	33.4 (2.3)	9.9 (1.8)	33.0 (3.9)
III LW	5.2 (0.9)	28.1 (2.9)	2.7 (0.9)	23.9 (3.5)
15°C	2.5 (0.7)	28.3 (2.2)	5.7 (1.0)	9.0 (2.0)
		(240 animals in 60 broods per estimate)		
After 3 years				
10°C	21.0 (4.4)	13.0 (3.7)	14.9 (3.6)	17.0 (5.8)
15°C	10.4 (2.6)	11.2 (3.5)	0.0 (2.2)	2.9 (1.7)
23°C	1.5 (1.4)	1.0 (2.6)	7.1 (2.9)	11.9 (3.1)
SW	1.7 (2.2)	14.0 (4.7)	7.7 (2.8)	7.1 (4.0)
LW	0.0 (1.7)	17.0 (5.8)	8.2 (2.5)	4.1 (2.6)
		(80 animals in 20 broods per estimate)		

The 10°C, 15°C and 23°C were constant environments; SW and LW cycled at 1°/day and 1°/week between 10° and 23°C. The first experiment was done with three pairings over a six month period.

TABLE III

Estimates of physiological variation in temperature tolerance in male and female progeny in five environments, by subtraction of genetic variance from phenotypic variance.

Envir.	Preacc.	Postacclimation	Preacc.	Postacclimation
After 1–1½ years	Male		Female	
I 10°C	2.9	0.0	13.0	11.2
23°C	9.9	31.0	11.2	17.5
II SW	15.4	18.9	8.8	0.9
15°C	2.9	9.4	15.5	20.2
III LW	9.2	5.4	14.2	20.4
15°C	10.7	15.2	8.2	28.5
			(240 animals per estimate)	
After 3 years				
10°C	12.1	17.8	13.9	33.5
15°C	10.4	19.0	23.5	13.6
23°C	12.5	25.3	19.7	13.7
SW	20.0	27.0	16.6	30.2
LW	18.0	33.4	13.5	21.0
			(80 animals per estimate)	

The 10°C, 15°C and 23°C were constant environments; SW and LW cycled at 1°/day and 1°/week between 10° and 23°C.

ances, assuming, as explained earlier, that the other two components of variance in temperature tolerance can be ignored, at least in constant temperatures. These estimates also include errors of estimation of the two variances from which they are derived. There is evidence of an effect of acclimation but little systematic effect of either culture environment or sex. Male progeny from the three year sampling from each environment were always more physiologically variable after acclimation. This was not the case for male progeny from the earlier sampling nor for female progeny at either sampling. However, in most instances physiological variation was greater in progeny after acclimation than before.

In two of the environments (SW and LW) the effect of acclimation on physiological variation was opposite in the two sexes at the 1–1½ year sampling, and opposite in two other environments (15°C and 23°C) at three years. The effects of acclimation at 1–1½ years and at three years can be compared for each sex and each environment. In more cases than not the effect of acclimation was reversed, particularly in the female progeny. For example, female progeny from the 23°C environment were more physiologically variable after acclimation in the early sample but less physiologically variable after acclimation in the later sample.

Physiological variability was also measured as the change in mean tolerance due to acclimation to a higher temperature (23°C) for 24 h. The average responses of progeny to the higher temperature are shown in Table IV. Again the sexes were comparable, environment by environment, in each sampling period. Exceptions were in the SW environment in both periods. In the earlier sampling male progeny from the SW regime acclimated less than female progeny, but the order was reversed, presumably due to further selection, in the later sampling. The responses of male and of female progeny at three years were lowest in the SW environment and were actually the two lowest of all ten mean responses.

TABLE IV

Average responses of individual progeny from five environments to acclimation at 23°C for 24 h, measured as difference in temperature tolerance before and after acclimation.

Environment		Male	Female
After 1–1½ years			
I	10°C	5.6 (3.7)	5.5 (4.6)
	23°C	5.9 (4.3)	6.2 (4.7)
II	SW	4.8 (4.3)	7.6 (4.4)
	15°C	4.0 (3.9)	7.3 (5.4)
III	LW	5.4 (4.2)	4.6 (5.1)
	15°C	4.4 (4.1)	3.7 (5.3)
		(240 animals per estimate)	
After 3 years			
	10°C	9.3 (5.1)	9.7 (6.6)
	15°C	8.9 (5.2)	9.6 (6.1)
	23°C	10.4 (4.7)	10.3 (6.1)
	SW	8.2 (6.1)	6.0 (5.9)
	LW	9.2 (7.4)	10.5 (5.3)
		(80 animals per estimate)	

The 10°C, 15°C and 23°C were constant environments; SW and LW cycled at 1°/day and 1°/week between 10° and 23°C. Standard deviations (not standard errors) are shown in parentheses.

Physiological variability or flexibility, measured as just described, itself varies genetically among individuals. Differences among individuals in their potential for physiological change are due partly to their different genotypes. Additive genetic variances in physiological flexibility are shown in Table V. There apparently were effects of sex, of time of sampling, and of environmental treatment.

Female progeny had more additive genetic variance in flexibility than did males at the 1–1½ year sampling but not at three years. Variances were lower at three years, with one exception. In the SW environment the variance in male progeny increased. Variance in the 15°C environment, on the other hand, had almost disappeared in both sexes at three years. In both the early and later samples genetic variance in flexibility in male progeny from the 23°C environment was not detected.

Data on other traits, as well as on mean temperature tolerances, as affected by the regimes, are shown in Table VI which is a summary of data given in more detail in Bradley (1982). In general, differences between animals randomly sampled from the regimes did not persist in progeny. Hence the differences were presumably largely environmental, specifically maternal. Egg production was lowest in the 23°C environment, intermediate in the SW and LW regimes. The animals in these latter regimes were all exposed to 23°C. Egg production levels among female progeny raised at 15°C did not differ between regimes.

Sex ratio is an important trait in that a higher proportion of males would decrease overall reproduction, given similar reproduction levels in females. The percentage of males was significantly greater in broods from cycling regimes than from the 23°C regimes. The greater proportion of females in 23°C would not be sufficient to compensate for the lower egg production in 23°C, assuming these figures are indicative of egg production and sex ratios in nature. The differences in sex ratio did not occur in progeny, so presumably were not the result of natural selection.

Viability, also an important component of fitness, is lowest in 23°C. The total number of broods sampled was also low in the 23°C regime, but of that total a higher proportion had no progeny than was the case in other regimes. The lower

TABLE V

Estimates, with standard errors, of additive genetic variance in degree of acclimation (when exposed to 23°C for 24 h) in male and female progeny from five environments on two occasions.

Environment			
After 1–1½ years		Males	Females
I	10°C	9.8 (1.4)	12.1 (2.0)
	23°C	0 (0.9)	9.9 (1.7)
II	SW	6.4 (1.4)	10.4 (1.5)
	15°C	5.8 (1.1)	11.8 (2.2)
III	LW	9.2 (1.5)	15.8 (2.4)
	15°C	6.1 (1.3)	6.3 (1.5)
After 3 years			
	10°C	4.9 (2.1)	7.8 (3.6)
	15°C	1.9 (2.0)	0 (2.4)
	23°C	0 (1.6)	6.3 (3.1)
	SW	16.3 (4.6)	2.1 (2.6)
	LW	6.6 (4.6)	3.1 (5.7)

The 10°C, 15°C and 23°C were constant environments; the SW and LW cycled at 1°/day and 1°/week between 10° and 23°C.

TABLE VI

Temperature tolerance of male and female progeny, egg production, sex ratio and viability in five temperature environments.

		10°C	15°C	23°C	SW	LW
Tolerance 0		17.3	16.2	16.7	17.4	15.8
Tolerance +		20.3	20.7	19.8	23.0	18.8
Animals per mean		80	80	80	80	80
Egg production:	Parents	33.1	33.6	11.1	24.3	24.9
	F_1	24.4	24.7	23.6	25.1	25.2
Sex ratio:	Parents	32.9	39.4	33.3	48.7	43.2
	F_1	39.1	48.1	43.2	46.9	42.9
Viability:	Parents	50.9	47.8	27.2	44.3	55.1
	F_1	30.9	36.1	30.0	33.5	37.5
No. of broods:	Parents	118	136	12	153	115
	F_1	41	47	31	39	46

The 10°C, 15°C and 23°C were constant environments; the SW and LW cycled at 1°/day and 1°/week between 10°C and 23° (Adapted from the original data in Bradley, 1982).

viability in 23°C broods did not persist to the F_1 generation, although broods originating in the 10°C and 15°C cultures differed significantly in viability.

The relationships between temperature tolerance and other fitness traits in the same broods were investigated with data collected for heritability estimates (Bradley, 1978a). The correlation matrix is shown in Table VII. There is little obvious evidence of negative relationships between temperature tolerance and egg production, viability, or sex ratio. Indeed the relationship between egg production and tolerance, free of viability effects, was actually positive. The standard partial regression coefficients (path coefficients) between tolerance and egg production were 0.48 and 0.21 for male and female brood averages, respectively. On the other hand, if the correlation between sex ratio (proportion of males) and tolerance is real, then the correlation with proportion of females, and so with reproductive output, would be negative.

DISCUSSION

There is some evidence that directional selection for temperature tolerance in the 23°C environment resulted in a reduction in genetic (and phenotypic) variation. The estimates of genetic and phenotypic variances in male progeny from the 23°C environment were lower than in the other environments at three years (Tables I and II). This agrees with the expectation that additive genetic variation will decrease as certain genotypes leave relatively more progeny and gene frequencies move away from intermediate levels. McLaren (1976) reported reductions in genetic variance in a number of demographic traits in the copepod *Eurytemora herdmani* as a result of selection.

On the other hand, in the cycling environments (SW and LW), where selection was actually more effective than in the 23°C environment according to the mean tolerances of randomly sampled progeny measured at two years (Ketzner and Bradley, 1982), additive genetic variance appeared to be maintained in males at the

Table VII

Correlations among number of eggs, number of adults, egg-to-adult viability, sex ratio, average tolerance of male progeny and average tolerance of female progeny in 51 broods.

	2	3	4	5	6
1. Egg no.	0.48	−0.39	0.02	0.11	0.39
2. Adult no.		0.44	0.12	0.18	0.24
3. Viability			0.27	0.17	0.04
4. Sex ratio				0.23	0.21
5. Mean tolerance[a] of female progeny					0.28
6. Mean tolerance[a] of male progeny					

[a] Measured as least squares deviation of female brood mean tolerance, distinct from male parent effect.

three year sampling. However, acclimation for 24 hours was necessary for the variation to be expressed (Table II).

Genetic variation in the female progeny from the various environments was maintained and expressed, with the possible exception of those from the 15°C environment at three years (Table II). These results contrast with previous results from progeny of wild populations, where female progeny consistently expressed much lower genetic variation than did male progeny (Bradley, 1978a). Such a change may have resulted from selection, or perhaps, although this is not a likely explanation, from inbreeding in the laboratory cultures.

The association of environmental and genetic variation has been demonstrated before in Mendelian traits such as enzyme variants (McDonald and Ayala, 1974), third chromosome inversions (Dobzhansky *et al.*, 1966) and coat color (Gill, 1977), and in quantitative traits such as skeletal development in mice (Garrard *et al.*, 1974) and bristle number in *Drosophila* (Beardmore, 1961; Gibson and Bradley, 1974).

Thus, there are precedents for exhaustion of genetic variance in a constant environment and its maintenance in a cycling environment, always assuming that temperature tolerance is under selection. Such an assumption seems valid given previous work demonstrating that temperature tolerance measured by the short-term assay used in this study is closely related to survival at high temperatures (Bradley, 1976).

The ability to alter temperature tolerance internally may also be important to survival. In the past we have shown that female *Eurytemora* can acclimate to higher temperatures to a markedly greater degree than can males (Bradley, 1978b). Females and males acclimated at 23°C for 24 h could be easily classified, without error, solely on their temperature tolerances. Such a clear sexual dimorphism was not observed in general in the present experiment, but neither were the responses of the two sexes generally the same in the different environments.

Two measures of physiological flexibility were used in this study. The measurements shown in Table III reflect random non-genetic variation in a constant 15°C environment and may not be related to the potential of the organism to adapt physiologically. The measurements in Table IV indicate the degree to which individuals can adapt in the short-term (24 h). In previous work both estimates were much higher in females (Bradley, 1978a,b) but this was not the case in the present study. In general, males and females were more alike in short-term change in mean tolerances (Table IV) than in their random physiological variances (Table III). Also, while the degree of acclimation was generally higher after three years (Table

III), neither total phenotypic (Table I) nor physiological variation (Table III) increased as much after acclimation at three years as either did at 1½ years. Thus it appears that random physiological variation and physiological response to stress are not related, at least at the population level.

There was evidence for selection for average response due to acclimation, the second measurement, when the figures at 3 years and 1½ years were compared (Table IV). The exception was the SW environment. The sexes were comparable in most environments, again except in the SW environment. Additive genetic variance seemed to be absent in male progeny at both samplings in the 23°C environment, and low in the 15°C environment after three years in both males and females. This may indicate a significant response to selection since the levels of acclimation (Table IV) were also high in the 23°C and relatively high in the 15°C environment. In males, but not in females, the average responses at three years and the additive genetic variances at three years were in reverse order by environment, again suggesting selection for physiological flexibility in males.

Why the sexes should behave differently in their response to selection is not clear, especially since in a sexually reproducing species there is 50% gene flow or exchange between the sexes each generation. One of the interesting features of this experiment is that the sexual dimorphism in expressed genetic and physiological variance disappeared, to be replaced by apparent dimorphisms in selection response and in the effect of acclimation on expressed variance.

The fact that the consistently higher additive genetic variance in males and consistently higher responses to acclimation in females were no longer observed has been discussed earlier. Some of the differences in selection response have also been mentioned, with more evidence for selection in male progeny. Further evidence that selection pressure is greater on males, or at least on genotypes expressed in males, comes from the genetic variances measured before acclimation in the 23°C, LW and SW environments (Table II), which were all low.

The effect of acclimation on the expressed variance in temperature tolerance also differed between the sexes, particularly in certain environments. Whereas acclimation increased phenotypic variation in temperature tolerance in all environments in both sexes at 1–1½ years, at the 3 year sampling the effects were opposite in the sexes in the 10°C, 15°C, and 23°C environments (Table I). Genetic variances at those years (Table II) were increased following acclimation in the 1–1½ year samples, but at 3 years the effect in the SW and LW environment was quite different in males and females. Incidentally, Ushakov *et al.* (1977) suggested that genetic distinction is reduced following acclimation. So our evidence both contradicts and supports this suggestion, dependent partly on whether the animals have been selected or not.

All of this again begs the question of how the sexual dimorphism in genetic variation and physiological variation is maintained in nature and how it was reduced, reversed, or even eliminated in the laboratory. The large residual genetic variances observed would seem to eliminate random drift in gene frequencies as an explanation, so the change must have occurred by selection and clearly by selection pressure quite different from that in nature.

Differential selection pressure between the sexes is one means by which genetic variance and the sexual dimorphism might be maintained (Bradley, 1982). However, the present data indicate that the former was maintained (or expressed) in females, but less so in males; and the latter changed in that males no longer expressed more genetic variance and actually expressed less than did females. Thus we could conclude that the dimorphism itself has genetic variation.

Another model, also suggested in the review (Bradley, 1982) states that directional selection for temperature tolerance occurs in the warmer temperatures and this selection is resisted by negative relationships with other fitness traits, such as egg production. Thus, as temperatures decreased, tolerance would decline because of selection for other traits. Egg production was lowest in the environments reaching 23°C (Table VI) in which tolerance was highest. However, the differentiation was not present in the F_1 progeny. Greater proportions of males were present in the SW and LW regimes, but again the difference disappeared in the progeny. Viability was lower in 23°C than in the cycling regimes and lower in the SW than in the LW regime. However, once again these differences were not present in the F_1 progeny. In summary, there are no obvious mean genetic differences between regimes in fitness traits other than temperature tolerance, but environmental effects on these traits may result in selection for temperature tolerance being less effective. Finally, there are no obvious relationships between mean tolerances and mean egg production, sex ratio or viabilities of progeny in the regimes.

There are no dramatic negative relationships between temperature tolerance and other fitness traits at the brood level either. In Table VII correlations are shown between temperature tolerances and four fitness traits. There is no evidence directly from the correlation matrix on negative relationships, nor when one performs a path analysis including egg production, viability and mean tolerance. In both male and female progeny the standard partial regressions of tolerance on egg production are positive (0.48 and 0.21 in males and females, respectively). If the relationship had been negative and genetic, then selection for tolerance would in effect be reversed when temperature stress declined, a form of genetic homeostasis proposed a long time ago by Lerner (1954). The argument by Lerner was in the context of artificial selection (being resisted by natural selection). The argument here would have been for a return of genes for high temperature tolerance to intermediate frequencies as a result of the highest fitness at lower temperatures of genotypes having intermediate temperature tolerances. So far, therefore, this model is not supported, although it should be noted that there may be a negative relationship between proportion of females and temperature tolerance at the brood level.

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SYSTEMATICS, GROWTH, AND DEVELOPMENT OF *LAFOEINA MAXIMA*(=*KERATOSUM COMPLEXUM*) (HYDROZOA, CAMPANULINIDAE)

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ABSTRACT

Keratosum complexum Hargitt, 1909, is a junior synonym of *Lafoeina maxima* Levinsen, 1893. Several specimens have been collected from the type locality of *K. complexum* and compared with Hargitt's description and type specimen. These were compared with Levinsen's holotype of *L. maxima* and with another specimen of *L. maxima* from Greenland.

L. maxima superficially resembles a finger sponge. It has slightly branching stems which consist of many parallel tubes from which arise the hydranths, gonangia, and nematophores. With a height of up to 15 cm and a thickness of as much as 5 mm, the stems represent an extreme example of a fascicled hydroid stem.

Dormant hydroids were collected in the summer of 1975 off Cape Cod. Hydranths and nematophores developed in the laboratory at 5–7°C.

Some specimens sent out simple stolons which anastomosed to produce a reticulum, and hydranths developed from them. This condition, I believe, imitates a stage in the early development of the species. Later the hydrorhiza becomes a complex tangle of stolons making a mat as much as 0.5 mm thick.

The site of collection, 41°37′N, is the most southern known for this northern species.

INTRODUCTION

Hargitt (1909) described as a new genus and species of hydroid, *Keratosum complexum*, from Massachusetts. Superficially, it resembled a finger sponge, rather than a hydroid. Before reaching Hargitt, the specimens had been referred to a sponge taxonomist and to a bryozoan authority. Hargitt chose the name *Keratosum* to suggest its resemblance to a sponge. His description was as complete as possible from preserved specimens which had been dormant when collected. I have been able to collect one good specimen and several poor ones from the type locality. Comparison of specimens, as detailed below, make it certain that this hydroid is the well-known northern species *Lafoeina maxima* Levinsen, 1893. By studying the growth and development of specimens I have been able to understand why descriptions vary considerably, and why there has been uncertainty in the proper systematic placement of Hargitt's *Keratosum*. More importantly, the pattern of growth which produces a massive structure quite unlike that of most hydroids can be understood; and some details can be added to what is already known only on the basis of studies of preserved specimens.

The following comments by Hargitt, Fraser, Broch, and Hirohito indicate the difficulties in determining whether Hargitt's hydroid is in fact conspecific with *Lafoeina maxima*.

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Hargitt (1909, p. 380): "In 1892 Levinsen described a hydroid from Greenland, which seemed to have much in common with the one here under review. He had described it as a new species under the genus *Lafoeina* Sars, 1873, naming it *L. maxima.* At first it was thought the present species was probably identical with it, but when one undertook to work out details of morphology it became *more or less certain* that it was not the same species, but that moreover, it could hardly belong to the same genus . . ." [Italics not in original.]

". . . it seems very sure that the present one must find different generic housing. For example, in Sars' (1873) genus the hydroid has a reticulate hydrorhiza, and Levinsen describes something of the sort for *L. maxima*, but in the present species while there may be comprised something of the sort, it would be more correct to describe the complex stem as arising from a dense sponge-like base, etc." [I wonder what else he had in mind.]

Fraser (1944, p. 173): "This species [Hargitt's] seems to have some general resemblance to *Lafoeina maxima*, but differs from it too greatly to be placed with this species, if reliance can be placed on the figures.

"Possibly because the material was obtained at such a time of the year that it was not in good condition for diagnosis, the description and figures are not definite enough to decide upon its affinities, or even to be quite sure that it is a hydroid."

In writing the above, Fraser contradicts his earlier (1927, p. 327) statement: ". . . good reasons for believing that Hargitt's specimens belong to it [*Lafoeina maxima*]."

Broch (1909, p. 165) wrote that ". . . there seems to be good reason for believing that Hargitt's specimens belong to it [*Lafoeina maxima*], although because the specimens were imperfect, some of the important features were not readily recognizable."

Our initial interest in trying to collect specimens of Hargitt's hydroid was a result of plans for a visit by Emperor Hirohito of Japan to the Woods Hole scientific community in October, 1975. He had commented on *Keratosum* as follows (Hirohito, 1967, p. 4):

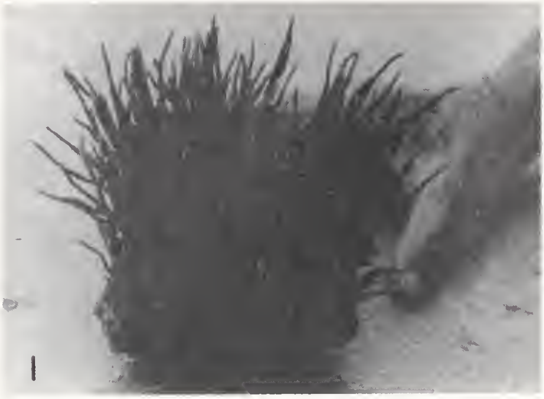
"The genus *Keratosum* may be assigned to the family [Clathrozonidae] as suggested by Stechow (1923, p. 59), because in this genus, which is represented by a single species, *K. complexum* (Hargitt, 1909, p. 379), the skeleton consists of an axial part made up of more or less parallel and anastomosing stolons and a peripheral part composed chiefly of hydrothecae and nematothecae. Nevertheless, the hydranths and gonothecae were missing from all of Hargitt's materials, so that the systematic position of the genus has remained undetermined to this day. Such being the case, the status of *Keratosum* is set aside from the present discussion."

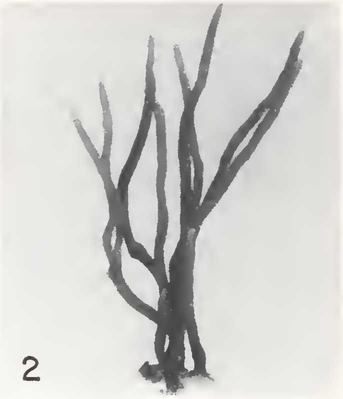
Indeed, Stechow (1923, p. 59) placed *Keratosum* in the family Clathrozonidae but added: "Systemat. Stellung unsicher!"

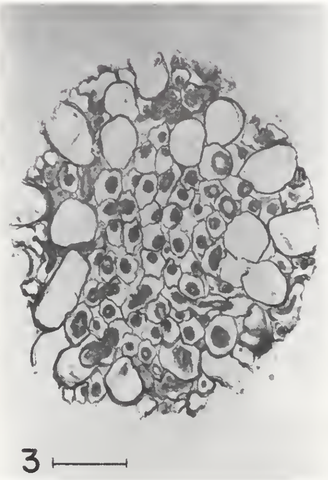
In a second paper, Hargitt (1911) decided that *Keratosum* belonged in the same family as *Clathrozoon wilsoni* Spencer, 1891. I mention this only to complete the record since the identity of *Keratosum* with *Lafoeina* makes Hargitt's proposal irrelevant. The family Campanulinidae, in which *Lafoeina* is placed, is distinguished from other families of thecate hydroids in having a radially symmetrical hydrotheca bearing an operculum. The operculum would not have been observable in the poor specimens which Hargitt studied.

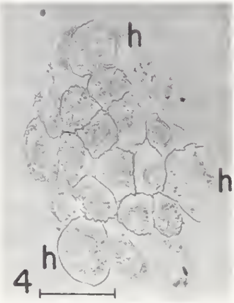
MATERIALS AND METHODS

The specimens used are described beyond. The most useful was dredged at Crab Ledge about 9 km east of Chatham, Cape Cod, Massachusetts, 41°37.4′N,









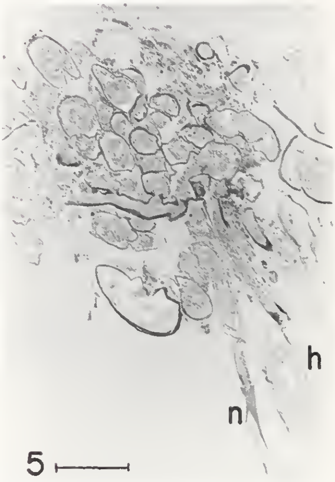


FIGURE 1. Crab Ledge 1975, specimen. Photographed soon after collection. The width of the rock at the base is 10 cm.

FIGURE 2. A part of the Crab Ledge, 1975 specimen. The height of the tallest stem is 8 cm.

FIGURE 3. Cross section of a stem of the Crab Ledge, 1975 specimen. Bar = 0.5 mm.

FIGURE 4. Cross section near the tip of one of the stems of the Holstensborg specimen. h, hydranth. Bar = 0.5 mm.

FIGURE 5. Cross section of a stem of the Holstensborg specimen. h, hydranth; n, nematophore. Bar = 0.5 mm.

69°49.1′W, 8 August 1975 (Fig. 1). Pieces of this specimen are in the Gray Museum of the Marine Biological Laboratory, Woods Hole, Massachusetts; in the collection of the Biological Laboratory, Imperial Household, Tokyo, Japan; in the United States National Museum of Natural History—Smithsonian Institution (No. 60352); and in the author's collection.

Soon after collection, many of the stems were removed from the rock; some were preserved in the next few days, others were placed in jars of sea water in a refrigerator. During transfer to Indiana University, 17–19 August, the jars were surrounded by ice, then put in a refrigerator at 5–7°C. Although the stems were dormant when collected, some of them later developed hydranths, nematophores, and stolonic outgrowths.

A good specimen was furnished through the kindness of Kay Petersen from the Zoologisk Museum, København. Data: Holstensborg, Greenland, 29 June 1908, Legit: "Tjalfe" st 88. Det. P. L. Kramp Journal 26-11-1908. Except for the Crab Ledge, 1975, specimen, this is the only one which shows developed hydranths. A small piece of it was sectioned.

I was lent a part of the holotype of Levinsen's (1893) specimen, also from the museum in Copenhagen. It was collected near Greenland. A small piece of this was sectioned and the rest returned.

J. S. Rankin gave me a specimen labeled: "Off Race Point, 190 feet [58 m], 9 July 1976, R/V Westward, 70°11'W, 42°08'N." Another specimen from Crab Ledge collected in July, 1977, was kept alive through the following fall. Although some buds developed in September none completed the formation of normal hydranths.

The National Museum of Natural History—Smithsonian Institution lent me the type of *Keratosum complexum* Hargitt "No. 42232, Crab Ledge, Mass. Sta 7835, 18 fms [32 m], by 'Fish Hawk' and R. C. Osburn on 2 September 1903 and 23 July 1907." It is presumed that Hargitt lumped the collections from both dates together. [The 1907 collection must have been at Station 7835, and 1903 collection at one of the stations 7603–7609 (Summer *et al.*, 1911, part 1, p. 203–204.)]

Specimens were fixed using Bouins followed by 70% ethanol. Routine paraffin methods were used for sectioning, followed by hematoxylin and eosin stains. The specimen used for the scanning electron micrographs was anesthetized using magnesium sulfate, fixed with gluteraldehyde, further fixed with osmium tetroxide, and coated with gold palladium.

Morphology

Stems and basal mat

My best specimen (Fig. 1) from Crab Ledge, 1975, was on the upper surface of a rock. The rock surface was completely covered by a dense mat about 0.5 mm thick. The mat or hydrorhiza consisted of a tangle of stolons and, as viewed from above, of openings of hydrothecae similar to those on the surface of the stems. There were more than 100 upright stems, some with a few branches which bent upward. Pieces removed from the rock (Fig. 2) resemble Hargitt's figure. The Holstensborg specimen has the same colony form. The tallest stems are 10 cm high, the thickest have a diameter of 3 mm at their bases. Shorter stems have diameters of 1–2 mm. Each stem tapers to a rounded tip. The color is a light tan near the tips and darker toward the bases. The surface of the stems seem fairly smooth since the hydrothecae and nematothecae had been worn down to a common level (Fig. 3). The stems feel spongy.

The specimen provided by Dr. Rankin consists of a shell of *Spisula*, 11 cm broad, on which are scattered about 40 stems. Several of the stems are in clumps with a common hydrorhiza, while other arise singly. Their height varies from 0.7 to 4 cm with an average of about 1.5 cm.

One of the Holstensborg specimens consists of a mat from which three stems arise. The length of the longest is 7.5 cm and at its thickest has a diameter of 5 mm. It seems to have been taken from a ribbed shell since there are grooves on the underside of the mat. Because this specimen had well-developed hydranths and nematophores its surface appears more shaggy than any of the others. The other Holstensborg specimen consists of a mat with four stems which are shorter and thinner than those of the larger specimen.

Stems from both Levinsen's (1893) and Hargitt's (1909) types resemble my specimens from Crab Ledge. The Crab Ledge, 1977 specimen is different only in that the tallest stem is 15 cm high, a value which is the extreme cited by Naumov (1969, p. 340) for the species.

Each stem consists of a central axis of parallel tubes. Each tube is like a stolon or hydrocaulus in consisting of an outer perisarc and an inner coenosarc enclosing a gastrovascular cavity.

The stems of most tall hydroids consist of several adherent hydrocauli and are said to be fascicled. *L. maxima* may be regarded as an extreme example of a fascicled hydroid stem.

The cortex of a stem consists of hydrothecae, nematothecae, and gonothecae. In addition, as more tubes develop they must occupy the spaces between the thecae which had developed earlier.

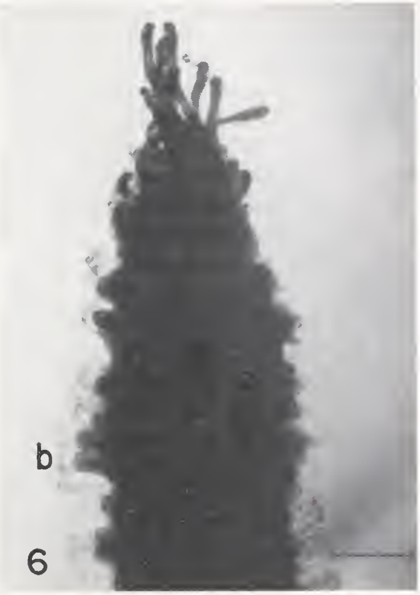
In a young stage when there are only a few tubes, the thecae extend freely from the stem as figured by Naumov (1969, p. 340) and by Fraser (1944, Plate 31, Fig. 146). Figure 4 is a section close to the tip of a stem of the Holestensborg specimen. Here there seem to be 14 tubes and three or four hydranths, one of which, at the tip of the section, is connected with a tube. Figure 5 shows an intermediate condition of a thicker stem of the Holstensborg specimen. Figure 3 is of a dormant stem from a Crab Ledge, 1975, specimen. The distal portions of the hydrothecae and nematothecae have been eroded but the proximal portions are rather deeply imbedded in the stem and the cortical region has a large number of tubes. In Figures 7 and 8 of a living specimen, only the most distal one third or one fourth of the hydrothecae extend beyond the surface of the stem.

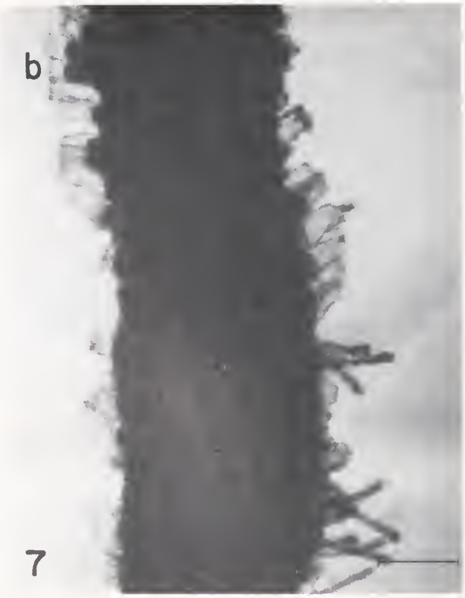
Hydranths and hydrothecae

The hydranths arise directly from the tubes. The hydrothecae are slightly narrower proximally and, as noted above, they may be free or imbedded through most of their length in the cortex of the stems. Naumov (1969) reports their height as 0.8–1.4 mm.

In the sections of Levinsen's type a few hydrothecae extend beyond the general surface of the stem and the longest measured is only 0.6 mm. Its real length cannot be determined since it may be partly eroded distally and its connection to its tube is missing in the sections. The sections of the Holstensborg specimen show a few hydranths whose hydratheca are 1.1 mm long. As with the Levinsen sections, none of mine show the full length of a hydrotheca; the longest measurement was 0.5 mm but the free region of the theca was wrinkled and the proximal region not in the section. An estimate of 0.8–1.0 seems reasonable.

The diameters of hydrothecae measured near the distal end have a range from 136–238 μm. Averages for Levinsen's: 140 μm; Holstensborg: 214 μm; Crab Ledge, 1975: 195 μm. I suspect that the lower value from the Levinsen sections may be because the material (after nearly 90 years) is somewhat shrunken. The slight difference between the Crab Ledge, 1975, and Holstensborg specimens is possibly real. Figure 7 shows several hydrothecae in profile and the variation in diameter can be seen, as well as variation in the angle with respect the the axis of the stem. Naumov reports that the hydrothecae are at an angle of 45–60° with the mouth toward the apex of the stem. Fraser's figure shows this feature as does Hargitt's.





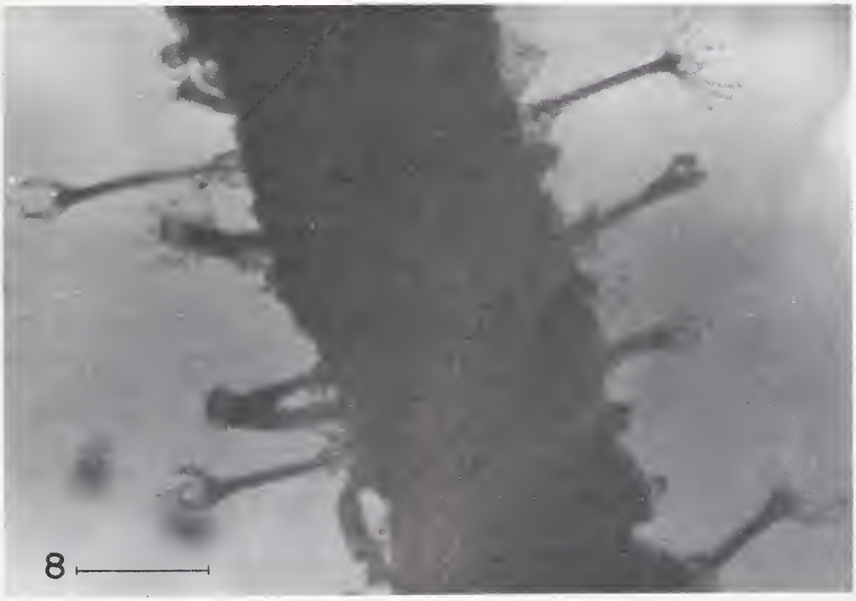


FIGURE 6. New growth of hydrocauli from the tip of a stem. 24 Sept. 1975. b, young hydranth buds. Bar = 0.5 mm.

FIGURE 7. Same stem as in Figure 6 with same hydranth buds and clusters of nematophores. Bar = 0.5 mm.

FIGURE 8. Hydranths, 20 Nov. 1975. Bar = 1 mm.

Near the tip of some of our active stems this feature is also seen, but in the more proximal regions of older and thicker stems, the hydranths and hydrothecae are nearly at right angles to the stem as shown in Figures 7 and 8.

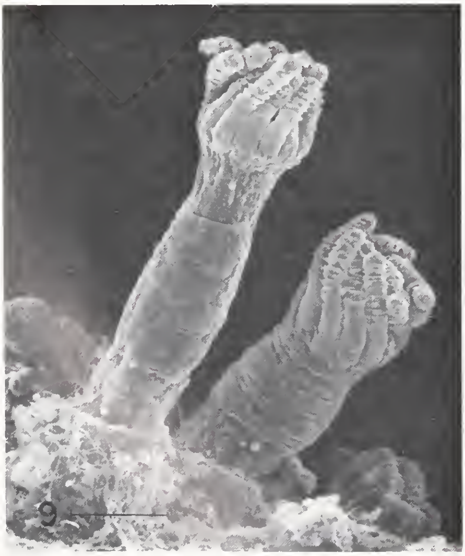
Operculum

The operculum consists of about 12 triangular plates or lappets which project outward from the circular opening of the hydrotheca. When first formed the plates are continuous with the wall of the hydrotheca. Only after they have folded is a definite base established. While the opercular plates are nearly equally long for each hydrotheca, they show considerable variation at their bases. One hydrotheca, for example, had plates whose bases varied in width from 26 to 53 μm. The length of measured plates ran from 170 to 205 μm. When the hydranth is not extended the plates meet at their tips to form a cone slightly taller than its base. They open when the hydranth is extended. Often, in empty hydrothecae, the plates have collapsed inward. Many hydrothecae lack the plates, but these are cases in which the hydranth itself has regressed. In poor specimens most hydrothecae have been worn or broken so that they do not project beyond the surface of the stem as in Figure 3.

The body of the hydranths can extend more than a millimeter beyond the hydrotheca (Fig. 8). The longest measured from mouth of a hydrotheca to base of tentacles was 1.4 mm. The extended tentacles are nearly 0.5 mm long. In the SEM picture (Fig. 9), both the tentacles and body are partly contracted.

Tentacles

The hydranths of our specimens had 12 tentacles. Although our records show a few counts of 11 or 13 this is likely due to the difficulty of observation since thecate hydroids as a rule have an even number of tentacles as shown by Berrill (1949). One of the sections of the Holstensborg specimen had been cut across the tentacles and an exact count of 16 was possible. Counts of tentacles of hydranths on the stems gave values close to 16. The difference between 12 and 16 may reflect a more favorable environment for development in the Holstensborg specimens. In



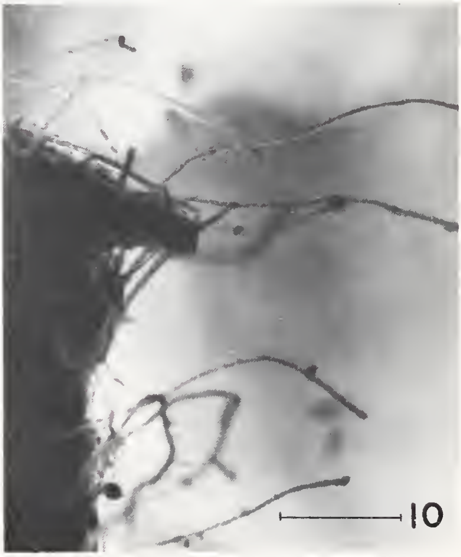


FIGURE 9. SEM showing two partly contracted hydranths. 24 Nov. 1975. Bar = 200 μm.
FIGURE 10. Stolons growing out from the basal disk. 20 Nov. 1975. Bar = 1 mm.

hydroids the number of tentacles in a species shows some variation so no systematic distinction should be made on the basis of small variations in tentacle number. For example, hydranths of *Clathrozoon wilsoni* have 12–16 tentacles (Hirohito, 1967), and the sketch of his Plate IV shows this variation among hydranths of the same stem.

Gonangia

I have found gonangia only in a few of the sections of the Holstensborg specimen. The diameter of the gonotheca is slightly more than 300 μm. The tissue is not satisfactory for critical examination. I can add nothing to Broch's (1909) statement: "In the gonotheca of the studied individuals there are several planula larvae, but the fixed material gave no detailed understanding of the gonangial condition." [My translation.]

Nematophores

These extend above the surface of the stem by about 750 μm. The nematophores of Naumov's (1969, p. 340) figure are about 700 μm long. Their thickness is slightly less than 100 μm, and they may or may not show slight enlargement at the tip. The nematocysts conform to the type called basitrichous isorhizas by Weill (1934). The capsules are from 14 to 20 μm long and about 4 μm wide. A few were successfully discharged. Measurements of the barbed region were 14–18 μm, and the longest thread 160 μm. The nematocysts are closly packed in the tip of the nematophore. In sections of two nematophores, I was able to count 48 in six successive sections, 38 in four successive sections, the actual number must be more than 50.

DEVELOPMENT

Following their collection on 8 August 1975, stems were removed from the rock, placed in separate glass jars which were kept in refrigerators except during transportation when they were surrounded by ice water. Some stems were at first kept in a cold room at 16–17°C, but by 16 September all were kept refrigerated at 5–7°C.

The photograph (Fig. 6) taken 24 September shows new growth of the tubes which constitute the central core of stems. Hydranth buds had appeared by this time, and the same two buds are seen in profile in Figures 6 and 7. Also, several nematophores had developed as shown in Figure 7. In the following days many more buds were produced. Some of these developed tentacles, but none emerged from the hydrothecae as fully developed normal hydranths. Whether tentacled or not, these buds regressed leaving empty hydrothecae, a few of which are shown in profile in Figures 6 and 7.

Some, but not all, of the stems produced normal hydranths. On 21 November normal hydranths were observed on one stem, and it and two other stems of the same specimen had normal hydranths through 12 December; after that date, no observations were made until 29 December. The records show no fully developed hydranths on 29 December nor during the next week; finally, however, there was a new burst of hydranth development which ran from 9 January through 13 January. After that time buds were observed but no fully developed hydranths.

Another specimen produced complete hydranths from 18 November to 24 November. Figure 8 shows some of the hydranths of this specimen on 20 November.

It again produced feeding hydranths from 29 December through 10 January. Thereafter, buds were produced which did not complete development.

Four other specimens produced normal hydranths during only one period. The dates are: 1–14 January; 6 January–10 February; 29 December–15 January; and 29 December–2 January.

I made only a few observations to determine the length of life of individual hydranths. Of four hydranths identified on 9 January, only one remained five days later. The others had regressed; that is, they had not merely withdrawn. While this regression was taking place, young buds were appearing nearby. In the few cases which have been studied closely, hydranths of thecate hydroids have been shown to regress after about a week; and frequently after a few days another hydranth is produced at the same location. An earlier paper (Crowell, 1953) on regression and replacement cites other investigations of this problem.

The basal mat or hydrorhiza consists of a mass of tangled stolons. Single stolons grew out from this in several of our animals (Fig. 10) and in one case attached to the side of the jar. Here they were not easily observed, but at the time when specimens were preserved, the jar was broken and the stolons could be examined as well as the hydrothecae which had arisen from them. It is clear that stolons often fuse so that the hydrorhiza is a true reticulum. Most of the stolons lack coenosarc in the preserved specimen. Most hydrothecae are empty and only a few undamaged. When a new colony of *L. maxima* is established, it must at first take this form. At that time it might easily be mistaken for *Lafoeina tenuis* Sars, 1873.

Levinsen distinguished *L. maxima* with its thick mat from *L. tenuis* with its simple reticulate stolon system. As indicated above, this distinction would not apply to young specimens. Levinsen points out that there are only 4–6 large nematocysts at the tip of each nematophore in *L. tenuis* while there is a large, rounded battery of very small nematocysts in the nematophore of *L. maxima.* While I obtained counts of nearly 50 nematocysts for nematophores of *L. maxima*, Sars' figures show only a few, and these are represented as twice the size of those that I measured. The tips of the nematophores of *L. tenuis* are conspicuously swollen, only slightly or not at all in *L. maxima.*

DISTRIBUTION

Naumov (1960, 1969, p. 340) says: "All Russian northern and Far Eastern Seas, Davis Strait, Norwegian and Greenland seas. In Sea of Japan occurring at threshold depths for this species." Vul'fius (1937, p. 72), in translation, says: "Bay of Peter the Great, 1600–900 m; south of the Rimskogo-Korsakova Islands, 410–366." A chart shows that the edge of the continential shelf south of the Bay and Islands is at about 42°25′N. At 42°10′N the depths are more than 1600 m. Therefore, for the western Pacific the most southern report at about 42°20′N is slightly farther north than Crab Ledge at 41°37′. In addition to our localities, there are two in the Gulf of St. Lawrence (Fraser, 1944), and several in northern Canada (Calder, 1970). I know of no records for the eastern Pacific.

Colton and Stoddard (1973) furnish tables of bottom temperatures. For the Crab Ledge location, maximum temperatures in January through April range from 2.9°C to 5.0°C, and maxima in June through November 8.2°C to 12.6°C. I agree with Hargitt's conjecture that the elevated summer and fall temperatures are responsible for the dormant condition of specimens collected in those seasons.

Acknowledgments

I have received assistance from so many people that it is impractical to name them all. Included would be the paid and the volunteer crew of the "expedition" of August, 1975, authorized by the Director of the MBL; members of the Gray Museum who provided facilities for handling the specimens; those who furnished other specimens; translators of Latin, Norwegian, Danish, and Russian; and several critics of the manuscript.

Several colleagues took the photographs for the figures: Fig. 1, Charles Wyttenbach; Fig. 2, Ray Ritz; Figs. 3, 4, & 5, David Dilcher and Carl Longstreth; Figs. 6, 7, 8, & 10, George Malacinski; and Fig. 9, Rudolph Turner.

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INHIBITORY EFFECT OF HISTAMINE ON THE RELEASE OF MELANIN-DISPERSING HORMONE IN THE FIDDLER CRAB, *UCA PUGILATOR*

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ABSTRACT

Histamine (HA), a stimulator of H_1 and H_2 receptors, produced dose-dependent inhibition of the melanin dispersion which normally occurs when fiddler crabs, *Uca pugilator*, are transferred from a white to a black background. The HA precursor L-histidine, and 4-methyl histamine (4-MeHA), an H_2 receptor agonist, also inhibited melanin dispersion. 2-Methyl histamine (2-MeHA), an H_1 receptor agonist, enhanced melanin dispersion. The inhibitory effects of HA and 4-MeHA were abolished by the H_2 receptor blocker metiamide but not by blockers of either H_1 receptors or alpha_1 adrenoceptors. Melanin-dispersing hormone (MDH) release is accomplished mainly by stimulation of alpha_1 adrenoceptors with norepinephrine appearing to be the neurotransmitter involved. The H_1 receptor blockers pyrilamine and SA-97 antagonized 2-MeHA. HA-induced inhibition of melanin dispersion was potentiated by the noradrenergic neuron blocker bretylium and the alpha_2 adrenoceptor agonist B-HT 933. HA did not significantly affect melanin dispersion in crabs pretreated with 6-hydroxydopamine which destroys catecholaminergic neuroterminals. None of these drugs affected the melanophores directly. On the basis of these and previously obtained results it is suggested that H_1 and H_2 receptors are present on norepinephrine neurons involved in triggering MDH secretion, and administered HA inhibits MDH release by decreasing impulse-mediated noradrenergic neurotransmission through stimulation of H_2 receptors.

INTRODUCTION

Migration of the melanin in the melanophores of the fiddler crab, *Uca pugilator*, is controlled by antagonistic hormones, a melanin-dispersing hormone (MDH) and a melanin-concentrating hormone (Carlson, 1935; Sandeen, 1950; Fingerman, 1956). Release of MDH in this fiddler crab appears to be triggered by noradrenergic neurotransmission (Hanumante *et al.,* 1980, 1981; Fingerman *et al.,* 1981; Hanumante and Fingerman, 1981; 1982a, b, c).

In mammals, at least, injected histamine (HA) inhibits impulse-mediated norepinephrine (NE) release, but whether HA has a physiological role in local regulation of neurotransmission has not yet been completely established (Westfall, 1980). HA has also been suggested to have a neurotransmittory role in stimulating the release of a corticotropin-releasing hormone (Allolio *et al.*, 1981) and a prolactin (Donoso and Banzan, 1980) in mammals. With respect to crustaceans, HA has until now only been reported to be present in the nervous systems of the crabs *Carcinus maenas* (Kerkut and Price, 1961; Clay, 1968; Huggins and Woodruff,

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Abbreviations: HA, histamine; MDH, melanin-dispersing hormone; 2-MeHA, 2-methyl histamine; 4-MeHA, 4-methyl histamine; NE, norepinephrine; 6-OHDA, 6-hydroxydopamine.

1968; Woodruff *et al.*, 1969) and *Cancer borealis* (Clay, 1968). However, the role of HA, if any, in the physiology of crustaceans remains unknown. Therefore, it was considered important from the point of comparative pharmacology to determine whether HA has any direct or indirect effect on the release of MDH in the fiddler crab, *Uca pugilator.* This aim was implemented by administering highly specific histaminergic agents and noting their effects on the degree of pigment dispersion in the melanophores of crabs kept either on a white or a black background throughout the experiment or of crabs transferred from a white to a black background or from a black to a white background at the time the drugs were injected.

MATERIALS AND METHODS

Adult male fiddler crabs, *Uca pugilator*, from the area of Panacea, Florida (Gulf Specimen Co.) were used. Their melanophores were staged using the system of Hogben and Slome (1931). According to their scheme, stage 1.0 represents maximal pigment concentration, stage 5.0 maximal dispersion, and stages 2.0, 3.0, and 4.0 the intermediate conditions. To facilitate comparison of responses of the experimental and control crabs, the mean responses of both groups were used to calculate "Response Indices" as defined by Hanumante and Fingerman (1982a). The Response Index is the difference between the means of the control and experimental groups. For example, if the mean melanophore stage of the control group was 2.8 and that of the experimental group 3.6, then the Response Index is +0.8, the "+" signifying the melanin of the experimental group was more dispersed than that of the control group. A "−" signifies that the melanin of the experimental group was less dispersed than that of the control group. Each value presented below is based on average melanophore stage of 10 intact experimental crabs or 10 experimental isolated legs. The same number of control crabs and isolated legs was used. The data were analyzed by means of Student's *t* test with significance set at the 95% confidence interval. Standard Errors of the Means were also calculated.

When assays were performed on intact crabs, their melanophores were staged at the time of injection and 15, 30, 60, 90 and 120 minutes thereafter. However, when assays were performed on isolated legs, the melanophores were staged only at the time the legs were removed from the crab (at which time the legs were perfused with the test or control solution) and 15, 30, 45, and 60 minutes thereafter. The melanophores in isolated legs of this crab remain responsive for at least 120 minutes (Herman and Dallmann, 1975). However, in order to make sure that the melanophores we used in our *in vitro* experiments would indeed also be viable, isolated legs were challenged with eyestalk extract (containing 1/3 eyestalk equivalent/leg), the eyestalk being the major source of MDH in *Uca pugilator* (Sandeen, 1950). The volume of solution injected into each intact crab was always 0.05 ml. Likewise, each isolated leg was perfused with 0.05 ml of the appropriate solution. When intact crabs were used, the melanophores seen through the cuticle on the anteroventral surface of the second walking leg on the right side were staged. When isolated legs were used, the second and third walking legs from both sides of the crabs were removed, the legs from the left side serving as controls for the legs on the right side that received the test solution, and the melanophores on the anteroventral surface of these isolated legs were likewise observed for staging. These experiments with intact crabs and isolated legs were performed at 24°C under an illumination of 1190 lx.

Six of the drugs used in the present investigation, namely, metiamide (N-methyl-N′ {2 [5-methylimidazol-4-yl) methylthio]ethyl} thiourea) (Smith, Kline and French), 2- and 4-methyl histamine dihydrochloride (Smith, Kline and French), bretylium tosylate (American Critical Care), SA-97 (homochlorocyclizine) (Eisai), and B-HT 933 (2-amino-6-ethyl-4,5,7,8-tetrahydro-6H-oxazolo-{5,4-d}-azepin dihydrochloride) (Boehringer Ingelheim) were generous gifts. In addition, rauwolscine hydrochloride (Roth), coryanthine hydrochloride (Sigma), HA (Sigma), pyrilamine maleate (Sigma), 6-hydroxydopamine hydrobromide (Sigma), and L-histidine monohydrochloride (Sigma) were used. The concentration used for each drug was always 20 μg/dose of the free compound in assays on both whole animals and isolated legs except in two experiments, where the responses to different doses of HA were determined and when 15 μg HA were combined with 20 μg metiamide. All the drugs were dissolved in Pantin's physiological saline (Pantin, 1934). To dissolve the metiamide, a drop of HCl (1.2 *M*) was added to the saline. Consequently, a drop of HCl (1.2 *M*) was also added to the control saline for the metiamide experiments. The rest of the controls received pure saline.

Experiments and Results

Effects of histaminergic agents on melanin migration

The aim of this series of experiments was to determine whether HA and other agents acting through histaminergic mechanisms affect melanin migration in the fiddler crab. Mammals have two types of HA receptors, called H_1 and H_2 (Douglas, 1980; Schwartz *et al.*, 1980; Polanin and McNeill, 1981). The two classes of HA receptors were revealed by the differential responses of experimental animals to histaminergic agonists and antagonists. Histidine is a precursor of histamine (Douglas, 1980). 2-Methyl histamine (2-MeHA) is a specific agonist of H_1 receptors (Ash and Schild, 1966; Douglas, 1980), whereas 4-methyl histamine (4-MeHA) selectively stimulates H_2 receptors (Black *et al.*, 1972; Durant *et al.*, 1975; Owen *et al.*, 1979; Douglas, 1980; Polanin *et al.*, 1981; Tenner, 1981). None of these drugs produced melanin dispersion in crabs initially having maximally concentrated pigment and which continued to be kept on a white background throughout the experiment (Table I). However, a preliminary experiment had revealed that HA inhibited melanin dispersion in crabs transferred from a white to a black background. The melanin of fiddler crabs disperses in specimens transferred from a white to a black background and concentrates following transfer from a black to a white background. An experiment was then performed to determine whether this effect is dose related. For this purpose, four HA concentrations, namely 5, 10, 15, and 20 μg/dose, were administered to crabs having maximally concentrated melanin initially. These crabs together with controls underwent a background change from white to black immediately after receiving the HA or saline. It is evident from Figure 1 that HA produced dose-dependent inhibition of melanin dispersion.

L-Histidine and 4-MeHA, like HA, also significantly decreased the rate of melanin dispersion in crabs initially having fully concentrated melanin and which were shifted from a white to a black background (Table II). In contrast, 2-MeHA significantly enhanced the rate of melanin dispersion in crabs that underwent a background change from white to black and whose melanin was initially maximally concentrated (Table II).

In crabs, initially having maximally dispersed melanin and which were kept in black pans throughout the experiments, HA, L-histidine, and 4-MeHA produced significant inhibition in melanin dispersion (Table I), but 2-MeHA kept the melanin from concentrating to the extent evident in the saline-injected control crabs. The

TABLE I

Response Indices of melanophores of crabs administered a drug and maintained throughout the experiment on a white (W) or a black (B) background.

		Time (minutes)				
Drug	Background	15	30	60	90	120
Histamine	W	−0.1	0.0	0.0	0.0	−0.1
	B	−0.8*	−0.8*	−0.7*	−0.9*	−0.7
L-Histidine	W	0.0	0.0	0.0	0.0	0.0
	B	−0.2	−0.6	−0.3	−0.3	−0.8*
2-Methyl histamine	W	0.0	0.0	0.0	0.0	0.0
	B	+0.4	+0.5	+0.6*	+0.6*	+0.4*
4-Methyl histamine	W	0.0	0.0	0.0	0.0	0.0
	B	−0.2*	−0.2*	−0.4*	−0.4*	−0.4*

+, melanin more dispersed in experimentals than in controls; −, melanin less dispersed in experimentals than in controls.

At the outset (0 minute time), response indices for all groups of crabs were 0.0.

* Statistically significant, $P \leqq 0.05$ as compared with controls.

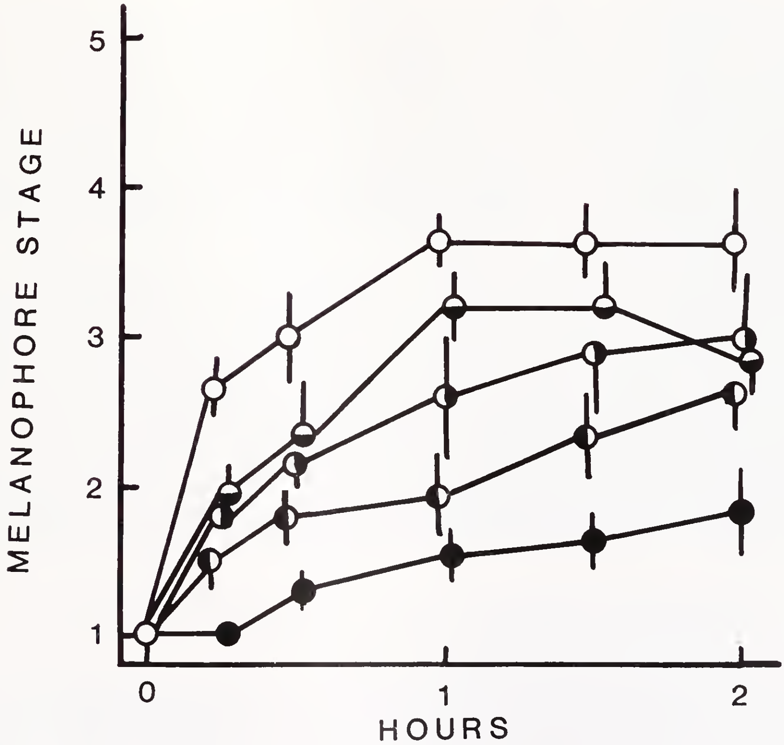

FIGURE 1. Relationships between melanophore stage and time for fiddler crabs, *Uca pugilator*, transferred from a white to a black background. Solid circles, crabs that received 20 μg histamine; circles half-filled on left, crabs that received 15 μg histamine; circles half-filled on right, crabs that received 10 μg histamine; circles half-filled on bottom, crabs that received 5 μg histamine; open circles, saline-injected controls. Vertical bars indicate SEM.

Table II

Response Indices of melanophores of crabs administered 20 μg of a drug and then changed from a white to a black background (W → B) or from a black to a white background (B → W).

		Time (minutes)				
Drug	Background	15	30	60	90	120
Histamine	W → B	−1.7*	−1.7*	−2.1*	−2.0*	−1.8*
	B → W	−0.3	−0.4	−0.1	−0.2	−0.5
L-Histidine	W → B	−1.2*	−1.1*	−1.4*	−1.0*	−1.0*
	B → W	+0.2	−0.1	−0.3	−0.3	−0.2
2-Methyl histamine	W → B	+0.1	+0.3	+0.6*	+0.3	+1.0*
	B → W	+0.2	+0.6*	+0.5	+0.6	+0.6*
4-Methyl histamine	W → B	+0.2	−0.7*	−1.1*	−1.2*	−1.6*
	B → W	−0.3	−0.5*	−0.6*	−0.3	−0.3

+, melanin more dispersed in experimentals than in controls; −, melanin less dispersed in experimentals than in controls.

At the outset (0 minute time), response indices for all groups of crabs were 0.0.

* Statistically significant $P \leqq 0.05$ as compared with controls.

melanin concentration observed in the control crabs maintained on a black background and whose melanin was initially in stage 5.0, was probably a result of excitement blanching caused by handling of the crabs when readings were taken and/or the circadian rhythm of color change (which fosters dispersion of the pigment during the daytime and concentration of the pigment at night), and/or the circatidal rhythm of color change which modulates this circadian rhythm.

HA and L-histidine did not significantly affect the rate of melanin concentration in crabs shifted from a black to a white background and whose melanin was initially maximally dispersed (Table II). However, 2-MeHA significantly slowed and 4-MeHA significantly increased the rate of melanin concentration in the crabs initially having fully dispersed melanin and which were shifted from a black to a white background. None of these histaminergic agents significantly affected pigment migration in the melanophores of isolated legs (Table III). On the contrary, MDH (eyestalk extract) was effective in keeping melanophores in isolated legs significantly more dispersed over saline-perfused legs (Table III), thereby confirming that our *in vitro* system was viable. Regardless of whether the melanin is maximally concentrated or maximally dispersed at the time a leg is removed, this pigment tends to attain an approximately intermediate degree of dispersion in isolated legs.

Effects of H_1 and H_2 receptor antagonists on melanophore responses to histamine and histamine agonists

The objective of this series of experiments was to characterize the receptors which mediate the HA-induced inhibition in melanin dispersion. Pyrilamine and SA-97 are specific H_1 receptor antagonists (Ash and Schild, 1966; Donoso and Banzan, 1980; Douglas, 1980; Gotow *et al.*, 1980), whereas metiamide selectively blocks H_2 receptors (Black *et al.*, 1972; Donoso and Banzan, 1980; Douglas, 1980). Crabs having maximally concentrated melanin initially and which underwent a

TABLE III

Response Indices of melanophores in isolated legs administered a drug or an eyestalk extract (⅓ eyestalk/crab).

Drug	Initial state of pigment	Time (minutes) 15	30	45	60
Histamine	MC	+0.1	+0.2	+0.1	0.0
	MD	0.0	0.0	0.0	−0.2
L-Histidine	MC	0.0	+0.3	+0.4	−0.2
	MD	−0.2	+0.1	+0.3	+0.2
2-Methyl histamine	MC	−0.4	+0.1	+0.1	−0.2
	MD	+0.1	+0.3	+0.3	+0.1
4-Methyl histamine	MC	−0.2	+0.1	+0.2	+0.2
	MD	0.0	+0.1	+0.1	0.0
Pyrilamine	MC	+0.2	+0.1	−0.1	−0.1
	MD	+0.2	−0.1	0.0	0.0
SA-97	MC	+0.2	+0.3	+0.2	+0.2
	MD	+0.1	+0.1	0.0	0.0
Metiamide	MC	−0.1	0.0	−0.4	−0.1
	MD	+0.1	0.0	−0.2	−0.1
Eyestalk extract	MC	+0.7*	+0.7*	+0.5	+0.8*
	MD	+0.5*	+1.2*	+1.4*	+1.7*

+, melanin more dispersed in experimentals than in controls; −, melanin less dispersed in experimentals than in controls.

At the outset (0 minute time), response indices for all groups of crabs were 0.0.

MC = Maximally concentrated, MD = Maximally dispersed.

* Statistically significant $P \leqq 0.05$, as compared with controls.

background change from white to black at the time of drug injection were used in this set of assays.

None of the H_1 and H_2 receptor blockers by themselves significantly affected melanin migration *in vivo* (Fig. 2A) or *in vitro* (Table III). Furthermore, the H_1 receptor blockers did not significantly antagonize the HA-produced decrement in the rate of melanin dispersion (Fig. 2B), but significantly decreased the 2-MeHA-induced increase in melanin dispersion (Fig. 3A). Of the two H_1 receptor blockers, SA-97 was more effective. Metiamide significantly overcame the HA and 4-MeHA-induced melanin concentration (Figs. 3B and 4A). However, the inhibitory effect of metiamide against HA while significant during the first 30 minutes of the experiment was not apparent after one hour.

Effects of noradrenergic agents on the histamine effects on melanophores

This series of experiments was devised to determine whether the HA-induced inhibition of melanin dispersion is due to interaction of HA with a noradrenergic mechanism. Several drugs were used. Coryanthine preferentially blocks $alpha_1$ adrenoceptors (Tanaka and Starke, 1980; Weitzell *et al.*, 1979; Timmermans *et al.*, 1981). Rauwolscine selectively blocks $alpha_2$ adrenoceptors (Tanaka and Starke, 1980; Weitzell *et al.*, 1979; Timmermans *et al.*, 1981) whereas B-HT 933 is a

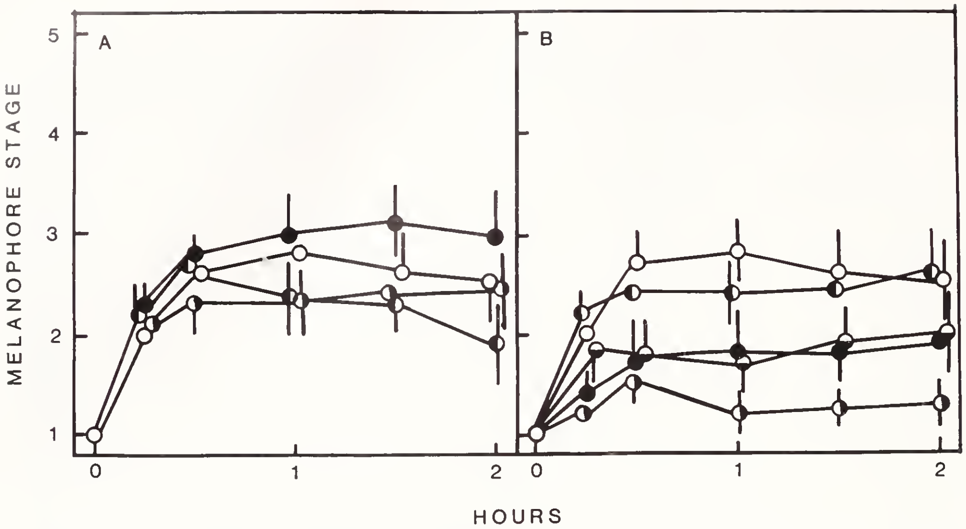

FIGURE 2. Relationships between melanophore stage and time for fiddler crabs, *Uca pugilator*, transferred from a white to a black background. (A): Solid circles, crabs that received metiamide; circles half-filled on left, crabs that received pyrilamine; circles half-filled on right, crabs that received SA-97; open circles, saline-injected controls. (B): Solid circles, crabs that received histamine; circles half-filled on left, crabs that received SA-97; circles half-filled on right, crabs that received histamine plus SA-97; circles half-filled on bottom, crabs that received histamine plus pyrilamine; open circles, saline-injected controls. Vertical bars indicate SEM.

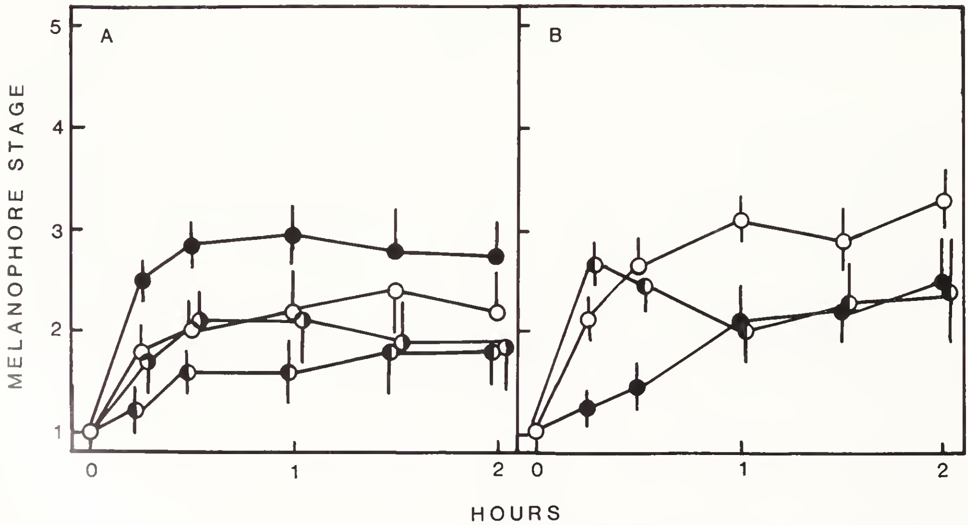

FIGURE 3. Relationships between melanophore stage and time for fiddler crabs, *Uca pugilator*, transferred from a white to a black background. (A): Solid circles, crabs that received 2-methyl histamine; circles half-filled on left, crabs that received 2-methyl histamine plus SA-97; circles half-filled on right, crabs that received 2-methyl histamine plus pyrilamine; open circles, saline-injected controls. (B): Solid circles, crabs that received histamine; circles half-filled on left, crabs that received histamine plus metiamide; open circles saline-injected controls. Vertical bars indicate SEM.

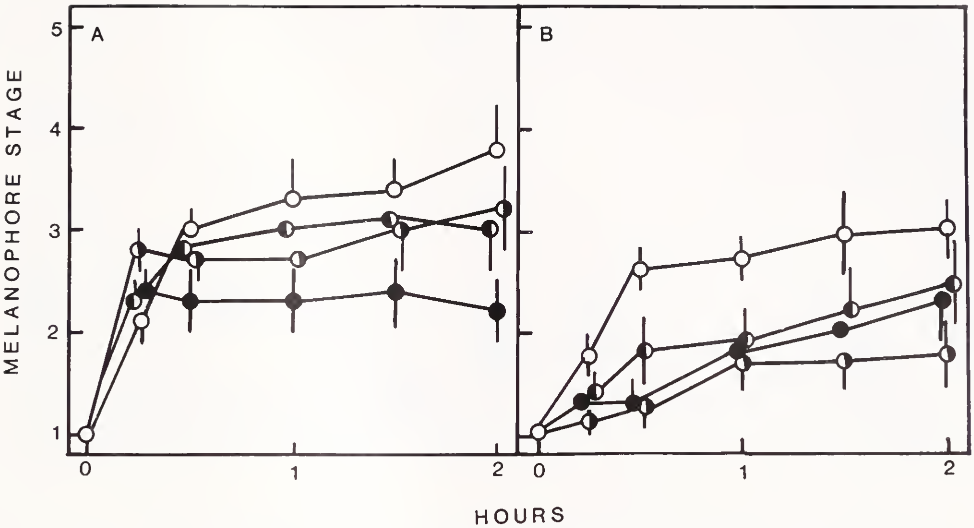

FIGURE 4. Relationships between melanophore stage and time for fiddler crabs, *Uca pugilator*, transferred from a white to a black background. (A): Solid circles, crabs that received 4-methyl histamine; circles half-filled on left, crabs that received metiamide; circles half-filled on right, crabs that received 4-methyl histamine plus metiamide; open circles, saline-injected controls. (B): Solid circles, crabs that received histamine; circles half-filled on left, crabs that received coryanthine; circles half-filled on right, crabs that received histamine plus coryanthine; open circles, saline-injected controls. Vertical bars indicate SEM.

highly selective $alpha_2$ adrenoceptor agonist (Kobinger, 1976; Hammer *et al.*, 1980; Timmermans and van Zwieten, 1980a, b; Pichler and Kobinger, 1981). Bretylium is a noradrenergic neuron blocker which specifically prevents impulse-mediated release of NE from presynaptic neurons (Boura and Green, 1959; Natoff and Dodge, 1980) whereas 6-hydroxydopamine (6-OHDA) selectively destroys catecholaminergic neuroterminals, including those of NE (Kostrzewa and Jacobowitz, 1974; Hwang *et al.*, 1980; Ritzmann and Bhargava, 1980). Also in this series of experiments crabs which were shifted from a white to a black background at the time of injection and whose melanin was maximally concentrated initially were used.

Coryanthine, by itself, like HA, significantly reduced the rate of melanin dispersion (Fig. 4B). The combination of HA and coryanthine also significantly decreased the rate of centrifugal melanin migration from that of the control crabs, but this decrement was not significantly less than that observed in the crabs that received either drug alone.

In order to obtain further support for the concept that HA-induced inhibition in melanin dispersion is mediated through H_2 receptors, the effects of 4-MeHA and coryanthine were determined. This combination reduced the rate of melanin dispersion which differed significantly from that of the control crabs as well as of the crabs that received either 4-MeHA or coryanthine. Each drug alone significantly reduced the rate of centrifugal melanin migration (Fig. 5A).

B-HT 933 (Fig. 5B) and bretylium (Fig. 6A) by themselves, like HA, kept the melanin less dispersed than in the respective control crabs. The combinations of HA and B-HT 933 (Fig. 5B) and HA and bretylium (Fig. 6A), further significantly

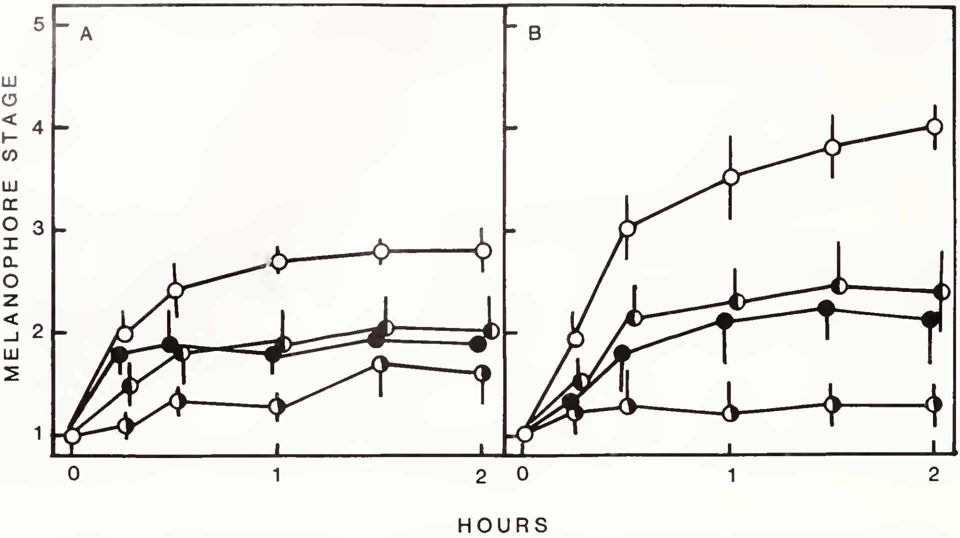

FIGURE 5. Relationships between melanophore stage and time for fiddler crabs, *Uca pugilator*, transferred from a white to a black background. (A): Solid circles, crabs that received 4-methyl histamine; circles half-filled on left, crabs that received coryanthine; circles half-filled on right, crabs that received 4-methyl histamine plus coryanthine; open circles, saline-injected crabs. (B): Solid circles, crabs that received histamine; circles half-filled on left, crabs that received B-HT 933; circles half-filled on right, crabs that received histamine plus B-HT 933; open circles, saline-injected controls. Vertical bars indicate SEM.

reduced the extent of melanin dispersion as compared with the crabs that received any of these drugs alone.

Rauwolscine significantly accelerated the rate of melanin dispersion (Fig. 6B). However, it did not affect significantly the HA-produced decrement in melanin dispersion when co-administered with HA.

6-OHDA was used as a pretreatment. This compound was injected into crabs with maximally concentrated melanin on a white background. One hour later these crabs were separated into two groups, one receiving HA and the other saline. In like manner, when the crabs were pretreated with the 6-OHDA, other crabs were pretreated with saline, and one hour later were divided into two groups, one that received HA and the other only saline. Immediately after receiving the second injection all of the crabs were transferred from the white background to a black one, and their melanophores were staged as usual for two hours. HA significantly inhibited melanin dispersion in the crabs pretreated with saline (Fig. 7). Likewise, the crabs that were pretreated with 6-OHDA and then received saline exhibited a significant reduction in their rate of melanin dispersion. On the other hand, HA did not significantly produce further inhibition of melanin dispersion in the crabs that had the 6-OHDA pretreatment.

DISCUSSION

The most logical hypothesis that can be deduced from the data presented above in light of the documented pharmacological actions of the drugs used herein in mammals is that HA H_1 and H_2 receptors are both present on NE neurons that control MDH release in the fiddler crab, *Uca pugilator*, and that administered HA

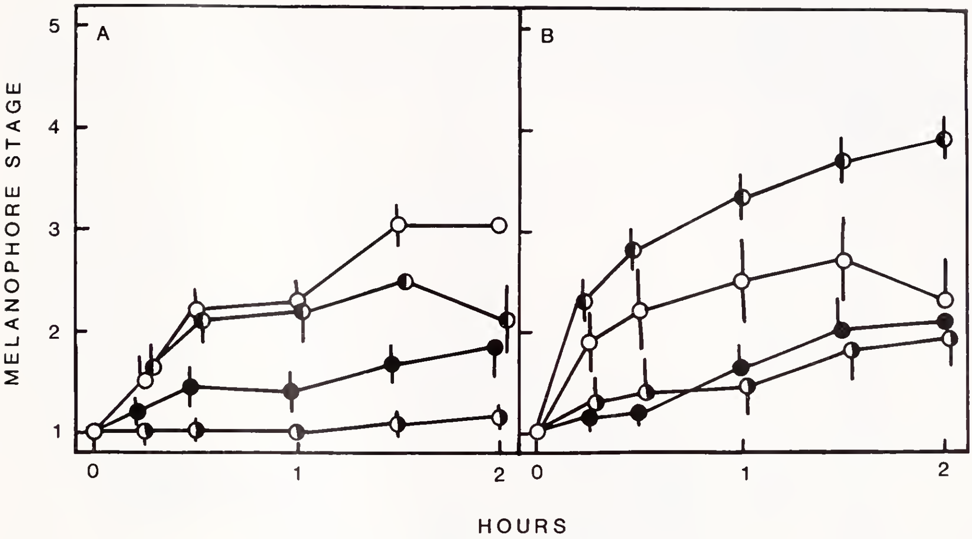

FIGURE 6. Relationships between melanophore stage and time for fiddler crabs, *Uca pugilator*, transferred from a white to a black background. (A): Solid circles, crabs that received histamine; circles half-filled on left, crabs that received bretylium; circles half-filled on right, crabs that received histamine plus bretylium; open circles, saline-injected controls. (B): Solid circles, crabs that received histamine; circles half-filled on left, crabs that received rauwolscine; circles half-filled on right, crabs that received histamine plus rauwolscine; open circles, saline-injected controls. Vertical bars indicate SEM.

inhibits MDH release by decreasing impulse-dependent noradrenergic neurotransmission through excitation of H_2 receptors. The effects of the drugs used herein are summarized in Table IV. The data presented in Figures 4B–7 also reinforce the hypothesis proposed earlier that NE triggers MDH release in this fiddler crab (Hanumante *et al.*, 1980). NE produces melanin dispersion in intact fiddler crabs but not in isolated legs. Consequently, the site of action of injected NE is considered to be the central nervous system. Furthermore, none of the histaminergic (Table III) or noradrenergic agents used in the present investigation affect melanin translocation directly (Hanumante and Fingerman, 1982b, c, and unpublished data), which is in keeping with their well proven effects on the nervous systems of other organisms and the noninnervated nature of fiddler crab chromatophores.

As stated earlier, at least in mammals HA receptors are classified into two types, called H_1 and H_2, depending upon their selective reactivity to specific histaminergic agonists and antagonists (Black *et al.*, 1972; Douglas, 1980; Schwartz *et al.*, 1980; Polanin and McNeill, 1981; Polanin *et al.*, 1981). For example, H_1 receptors are stimulated by 2-MeHA and 2-thiazolylethylamine and blocked by mepryramine and SA-97, whereas 4-MeHA and impromidine selectively stimulate H_2 receptors, and cimetidine and metiamide selectively antagonize H_2 receptors. Several recent reports reveal that in mammals HA can inhibit noradrenergic neurotransmission by an action on H_2 receptors located presynaptically on NE neurons (McGrath and Shepherd, 1976; Lokhandawala, 1978; Westfall, 1980; Vanhoutte *et al.*, 1981).

The absence of any melanin dispersion in the crabs administered the histaminergic agents and maintained throughout the experiment in white pans (and whose melanin was initally maximally concentrated) (Table I) is consistent with both the ability of a white background to foster melanin concentration and the pharma-

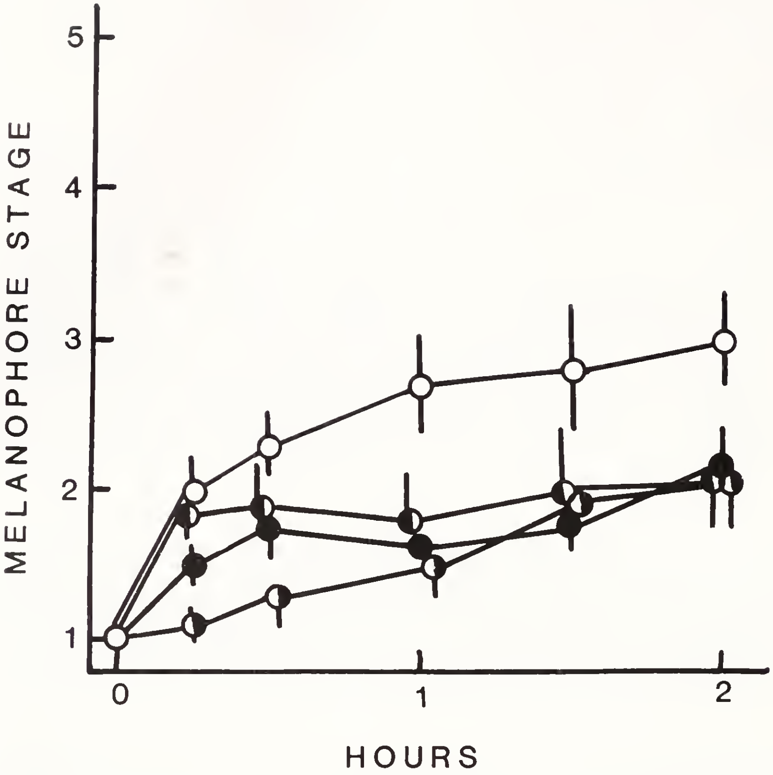

FIGURE 7. Relationships between melanophore stage and time for fiddler crabs, *Uca pugilator*, transferred from a white to a black background. Solid circles, crabs that received a 6-hydroxydopamine pretreatment followed by histamine; circles half-filled on left, crabs that received a pretreatment of 6-hydroxydopamine followed by saline; circles half-filled on right, crabs that received a pretreatment of saline followed by histamine; open circles, crabs that received two successive injections of saline alone (controls). Vertical bars indicate SEM.

cological actions of these drugs. The significant inhibition in the rate of centrifugal melanin migration produced by HA (an agonist of H_1 and H_2 receptors) and 4-MeHA (an agonist of H_2 receptors alone) in the crabs having maximally concentrated melanin initially and which underwent a background shift from white to black (Figs. 1, 4A, Table II) appears to be due to activation of H_2 receptors located on NE neurons. A black background, as stated above, fosters melanin dispersion, which will be effected by MDH. Consequently, on transferring the crabs from a white to a black background, neurogenic NE release would be initiated and/or increased which in turn would evoke MDH release by activating mainly alpha$_1$ adrenoceptors (Hanumante and Fingerman, 1981, 1982a). However, stimulation of H_2 receptors by HA and 4-MeHA would decrease stimulus-coupled NE secretion, and in turn less MDH would be released into the hemolymph. In mammals and the marine mollusk, *Aplysia californica*, HA is synthesized from L-histidine by L-histidine decarboxylase (Weinreich and Yu, 1977; Schwartz *et al.*, 1980). Presumably this enzyme required for the biosynthesis of HA is also present in the fiddler crab and the inhibitory effect of L-histidine on melanin dispersion (Table

TABLE IV

Summary of pharmacology and effects of the drugs used herein on melanin dispersion within melanophores of Uca pugilator *that underwent a background change from white to black.*

Drug	Pharmacology	Effect on melanin dispersion
Histamine	Stimulation of H_1 and H_2 receptors	−
2-Methyl histamine	Stimulation of H_1 receptors	+
4-Methyl histamine	Stimulation of H_2 receptors	−
SA-97, Pyrilamine	Blockade of H_1 receptors	None
Metiamide	Blockade of H_2 receptors	None
L-Histidine	Histamine precursor	−
Coryanthine	Blockade of α_1 adrenoceptors	−
B-HT 933	Stimulation of α_2 adrenoceptors	−
Rauwolscine	Blockade of α_2 adrenoceptors	+
Bretylium	Noradrenergic neuron blockade	−
6-Hydroxydopamine	Destruction of catecholamine neuroterminals	−

None of the drug significantly affected melanin translocation *in vitro.*
+ = Potentiation of melanin dispersion; − = inhibition of melanin dispersion.

II) in the crabs was due to the decarboxylation of this compound to HA which in turn stimulated H_2 receptors.

The end-organ responses produced by H_1 and H_2 receptors, when both are present on the same cell, are usually antagonistic to each other (Donoso and Banzan, 1980; Ganong, 1980; Gotow *et al.*, 1980; Geller, 1981). For example, stimulation of H_1 receptors increases plasma adrenocorticotropic hormone (ACTH) and corticoids whereas H_2 receptor activation has an inhibitory effect on the release of ACTH and consequently of corticoids (Ganong, 1980). In this context, H_1 receptors in *Uca pugilator* appear to be facilitatory, that is, their stimulation enhances stimulus-coupled NE secretion. In the crabs which underwent a background change from white to black and whose melanin was initially maximally concentrated, 2-MeHA an agonist of H_1 receptors, produced increased melanin dispersion (Table II, Fig. 3A), presumably by inducing increased NE secretion by stimulating H_1 receptors which in turn would produce a greater amount of MDH release into the hemolymph. However, when HA, which as stated above can stimulate both H_1 and H_2 receptors, was injected, the H_2 receptor-mediated inhibition on NE release apparently predominated because HA produced decreased melanin dispersion.

In the crabs maintained throughout the experiment on a black background and whose melanin was initially maximally dispersed, impulse-mediated NE release (and in turn MDH secretion) would be occurring at a greater rate than in crabs on a white background. Consequently, the H_1 receptor agonist 2-MeHA would facilitate stimulus-coupled NE release whereas H_2 receptor agonists such as 4-MeHA would inhibit stimulus-coupled NE release, thus accounting for the results presented in Table I for these substances, 2-MeHA fostering melanin dispersion in crabs on a black background and 4-MeHA reducing melanin dispersion.

In crabs having maximally dispersed melanin and which are transferred from a black to a white background, presumably the impulses calling for NE-mediated MDH release are terminated or reduced. Consequently, HA and its precursor L-histidine would be expected not to diminish NE-coupled MDH secretion in crabs transferred from a black to a white background as in crabs transferred from a white to a black background, which was the case (Table II). The small but significant decrease in the rate of melanin concentration produced by 2-MeHA and the increase produced by 4-MeHA were presumably due respectively to rapid increment (through H_1 receptors) or decrement (through H_2 receptors) in impulse-mediated NE release just before the appropriate melanin-concentrating events were initiated following the background change from black to white (Table II).

The inability (Fig. 2A) of the H_1 receptor blockers pyrilamine and SA-97 and the H_2 receptor blocker metiamide to affect melanin translocation when administered alone in a concentration (20 μg/dose) which significantly affected administered H_1 and H_2 receptor agonists (Figs. 2–4A) is not a unique phenomenon. In dogs H_1 and H_2 receptor antagonists did not alter the response to sympathetic nerve stimulation, even though injected HA produces an inhibitory effect on such noradrenergic neurotransmission by an action on H_2 receptors (Lokhandawala, 1978). Similarly, in dogs a dose of mepyramine (pyrilamine) that blocked the stimulatory effect of intraventricularly injected HA failed by itself to produce a significant decrease in ACTH and plasma corticoids (Rudolph *et al.*, 1979).

The H_1 receptor blockers, when co-administered with HA, were unable to abolish HA-induced melanin inhibition (Fig. 2B). This indicates that receptors other than the H_1 type are involved in the HA-mediated melanin inhibition. The data presented in Figure 3A substantiate this interpretation, revealing not only that H_1 receptor stimulation by 2-MeHA produced increased melanin dispersion but also that H_1 receptor blockers significantly prevented the usual increase in melanin dispersion in response to 2-MeHA administration. Metiamide significantly blocked the actions of HA and 4-MeHA (Figs. 3B and 4A), thereby indicating that H_2 receptors mediate the HA inhibition of melanin dispersion in fiddler crabs transferred from a white to a black background.

The data in Figures 4A–6A clearly demonstrate that administered HA is not interacting with the postsynaptic alpha$_1$ adrenoceptors, through whose activation NE mainly elicits MDH release (Hanumante and Fingerman, 1981, 1982a), or with the presynaptic alpha$_2$ adrenoceptors which regulate local NE release (Fingerman *et al.*, 1981; Hanumante *et al.*, 1981; Hanumante and Fingerman, 1981, 1982c). Coryanthine, the alpha$_1$ adrenoceptor blocker, antagonizes at least partially the MDH-releasing action of NE, the secretion of which would be initiated or augmented because of a background change from white to black. Therefore, in control crabs the melanin would disperse at a greater rate than in coryanthine-administered crabs, as seen in Figures 4B and 5A. In the crabs which received the combination of HA and coryanthine, not only would coryanthine, but, as discussed above, HA would also ultimately diminish MDH secretion. In the case of the crabs co-administered 4-MeHA and coryanthine (Fig. 5A), the combination of alpha$_1$ adrenoceptor-blocking and H_2 receptor-stimulating drugs presumably produced such a strong reduction in MDH release that the extent of melanin dispersion was significantly less than in the crabs injected with 4-MeHA alone.

The pharmacological effects (Fig. 5B) of B-HT 933, an alpha$_2$ adrenoceptor agonist, are also consistent with the earlier reports from this laboratory (Hanumante and Fingerman, 1981, 1982c). Presynaptic alpha$_2$ adrenoceptors have been implicated in a local negative feedback mechanism regulating release of NE, which, as stated above, triggers MDH secretion in *Uca pugilator.* Agonists of alpha$_2$ adrenoceptors diminish and antagonists of these adrenoceptors enhance impulse-dependent NE release (Langer, 1977; Starke, 1977; Vanhoutte *et al.*, 1981). The combined treatment of B-HT 933 and HA (Fig. 5B) produced further significant reduction in the extent of melanin dispersion over that induced by either drug alone. Furthermore, rauwolscine significantly offset the B-HT 933-induced decrement in the rate of melanin dispersion (Hanumante and Fingerman, 1982c) but not the HA-induced decrement (Fig. 6B). These observations unambiguously reveal that even though B-HT 933 and HA evoke similar end-organ responses in the fiddler crab, namely inhibition of melanin dispersion, the mechanisms of action of these agents are quite dissimilar; the former stimulating $alpha_2$ adrenoceptors and the latter exerting its effect by stimulating H_2 receptors, both receptors being situated presynaptically on NE neurons.

Bretylium, the noradrenergic neuron blocker which decreases action potential-linked NE release, slows the rate of centrifugal melanin migration in fiddler crabs having maximally concentrated melanin initially and which undergo a background change from white to black (Hanumante and Fingerman, 1982b). The fact that HA when co-administered with bretylium (Fig. 6A) further significantly decreased the rate of centrifugal melanin migration shows that these two drugs can act synergistically to inhibit melanin dispersion even though their mechanisms of action are different. Furthermore, HA does not enhance neuronal uptake of NE to ultimately reduce the rate of melanin dispersion nor does it affect other neurotransmitters known to be involved in triggering the release of other chromatophorotropic hormones in the fiddler crab (unpublished data). Therefore, we are left with the hypothesis mentioned earlier that H_2 receptor activation is responsible for the HA-mediated inhibition of melanin dispersion in crabs having maximally concentrated melanin initially and undergoing a background change from white to black.

6-OHDA inhibits the rate of melanin dispersion in fiddler crabs, *Uca pugilator*, having maximally aggregated melanin initially and transferred from a white background to a black one (Hanumante and Fingerman, 1982b). The fact that of the possible neurotransmitters tested at 8 μg/dose (including octopamine, NE, dopamine, and epinephrine) by Fingerman *et al.* (1981) only NE induced melanin dispersion in the fiddler crab strongly suggests that the inhibition of melanin dispersion produced by 6-OHDA was due to its destructive action on noradrenergic terminals. This, together with the failure of HA to further reduce significantly the rate of centrifugal melanin migration in crabs pretreated with 6-OHDA (Fig. 7), clearly indicates that the integrity of noradrenergic nerve terminals is essential for HA to exert its effect. That is, the H_2 receptors through whose activation HA produced an inhibition of melanin dispersion must be situated presynaptically on NE neurons.

It is interesting from the point of evolution and comparative pharmacology that in the marine mollusks *Aplysia californica* (Carpenter and Gaubatz, 1975; Gruol and Weinreich, 1979) and *Ochidium verruculatum* (Gotow *et al.*, 1980) two types of HA receptors corresponding to but not exactly the same as the mammalian H_1 and H_2 receptors, have been reported. For example, two temporally and ionically dissimilar hyperpolarizing responses were produced in *Aplysia californica* when HA was applied to neurons in the cerebral ganglion. The use of H_1 and H_2 receptor agonists and antagonists revealed that two distinct HA receptor types mediated these hyperpolarizing responses. However, the responses of these neurons to H_1 and H_2 reagents often differed from what has been found with mammals; the drugs were often non-selective or ineffective in *Aplysia.* In view of this difference between molluscan and mammalian histaminergic receptors and the need for due caution in interpretation of the data on invertebrate neuronal mechanisms obtained using agents specific for mammalian nervous systems, it would be worthwhile to completely characterize the HA receptors in *Uca pugilator* (*i.e.*, pharmacologically, biochemically, and physiologically) in order to determine their similarity (or dissimilarity, if any) to those of the mammals and other invertebrates.

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THE BIOLOGY OF A BROODING SEASTAR, *LEPTASTERIAS TENERA*, IN BLOCK ISLAND SOUND

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Abstract

Leptasterias tenera (Stimpson) was sampled from a muddy habitat, 28–37 m deep, dominated by tubicolous amphipods. The seastars were seldom aggregated and had mean densities of 3–8 individuals per m^2. *L. tenera* is holophagous, feeding primarily on small crustaceans which they capture with the pedicellariae and tube feet. Approximately half of the branched testes in mature males are degenerated or absent, but females usually have 10 lobate ovaries. Females initially brood embryos in their pyloric stomach and hold them externally during the latter half of the brooding period. The embryos are attached in cohesive masses by their elongate brachiolar appendages. *L. tenera* is slow-growing and iteroparous. The gonad and digestive caecum organ indices of *L. tenera* are not in phase with each other. Caecum index values indicate that females neither store more nutrient, nor utilize more reserves during the brooding period than males. Sexually mature males appear to have a higher rate of mortality than females. Thus, additional parameters must be examined to determine whether there is an energetic cost attributable to brooding adaptations in *Leptasterias*. It is possible that brooding specializations, small size, and slow growth rates typical for *Leptasterias* species are "cold adaptations" (*sensu* Clarke, 1980) rather than a coadaptive consequence of competition as suggested by Menge (1975) for *Leptasterias hexactis.*

Introduction

The genus *Leptasterias* Verrill contains over 30 species of generally small, 5 or 6 armed seastars which brood their young. The genus is restricted to the North Atlantic, North Pacific and the Arctic Oceans (Fisher, 1930; Djakonov, 1938). The life histories of several intertidal *Leptasterias* species have been examined (Chia, 1966, 1968a, b, 1969; Smith, 1971; Menge, 1972, 1974, 1975; O'Brien, 1976). This report on *Leptasterias tenera* (Stimpson) is the first ecological study of a subtidal species, although its reproductive cycle has been discussed in detail (Worley, *et al.*, 1977).

L. tenera is found along the eastern coast of North America from southwest Greenland to Cape Hatteras, North Carolina at depths from 18 to 230 m (Mortensen, 1927, 1932; Gray, *et al.*, 1968; Worley, *et al.*, 1977; Franz, *et al.*, 1981). The population of *L. tenera* studied occurred at depths of 28-37 m off the western side of Block Island. This is an area of muddy, fine sand with extensive patches dominated by "carpets" of tube-building ampeliscid amphipods and occasional mounds of bivalve shells. This subtidal, soft-bottom habitat of *L. tenera* contrasts markedly with the rocky environments of those intertidal *Leptasterias* species previously studied. Certain specializations of *L. tenera*, discussed below, include their

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holophagous feeding habits and their alternation of internal and external brooding during the initial and final segments of the brooding period.

MATERIALS AND METHODS

From 1971 to 1972 *L. tenera* was dredged monthly in Block Island Sound, Rhode Island, U. S. A., between 41° 11.4′ N, 71° 38.3′ W (Station C) and 41° 12.1′ N, 71° 36.2′ W (Station D). Specimens for dissection were transferred immediately to the laboratory in aerated, refrigerated water and others were preserved in seawater formalin for analysis of stomach contents. Small specimens were collected by washing dredge or grab haul sediments on 0.85 mm or 1.0 mm sieves.

All seastars were measured to determine a radius (R), the distance from the center of the disc to the tip of the longest arm. Organ indices, ratios between the weight of an organ system and the weight of an organism (Giese and Pearse, 1974), were used to describe quantitatively the dynamics of the reproductive cycle. For organ index measurements, whole specimens, freshly dissected gonads, and digestive caeca were drained and wet weighed. Organ indices were determined for 5 males and 5 females each month.

The density and dispersion of *L. tenera* were estimated from bottom photographs, each encompassing a 1.39 m^2 quadrat. Photographs were taken randomly in August 1970 and December 1971 using an automatically triggered benthic camera system towed across the study area. Dispersion was analyzed using the nearest-neighbor method of Clark and Evans (1954) as outlined in Poole (1974), a method which has been applied to the analysis of aerial photographs by Miller and Stephen (1966). The average distance between individuals and their nearest neighbors ($\bar{r}$), determined from the photographs, was compared with the expected distance (E_r) assuming a random distribution. The ratio (R) between observed ($\bar{r}$) and expected (E_r) values is equal to 1.0 if the seastars are randomly dispersed. Aggregated populations have values of R between 0 and 1.0, and regularly dispersed populations have values between 1.0 and 2.1496. The significance of the deviation from the expected value of R is determined using a standardized variate of the normal curve, $Z = \bar{r} - E_r/SE(\bar{r})$, where SE ($\bar{r}$) is the standard error of the mean observed nearest-neighbor distance (Poole, 1974).

Feeding periodicity and prey diversity were determined by analysis of stomach contents. The prey capture behavior of *L. tenera* from Cape Cod Bay was observed in running seawater aquaria at the Woods Hole Oceanographic Institution between March and June, 1974.

RESULTS

Density and dispersion

Dredging and photography showed that *L. tenera* was the most conspicuous and numerous epifaunal, predatory macroinvertebrate in the Block Island Sound Basin. Other predators such as the crab *Cancer borealis* Stimpson and the seastars *Asterias forbesi* (Desor) and *Asterias vulgaris* Verrill were present. The density of *L. tenera* in the study area ranged from 0 to 12 individuals per m^2, with means of 3–8 per m^2 (Table I). Of the 20 bottom photographs analyzed to determine dispersion (Table II), 15 indicated patterns which did not differ significantly from random dispersion. Five photographs indicated moderate to small, though statistically significant, deviations from randomness, toward aggregated patterns.

TABLE I

Density of Leptasterias tenera *in Block Island Sound based on the analysis of random underwater photographs.*

	Station C 9 XII 1971	Station C 5 VIII 1970	Station D 9 XII 1971
No. photographs	8	5	12
$\bar{x}/m^2$	4.80	7.97	3.32
Range/m^2	1.45–10.14	5.07–11.59	0–7.97

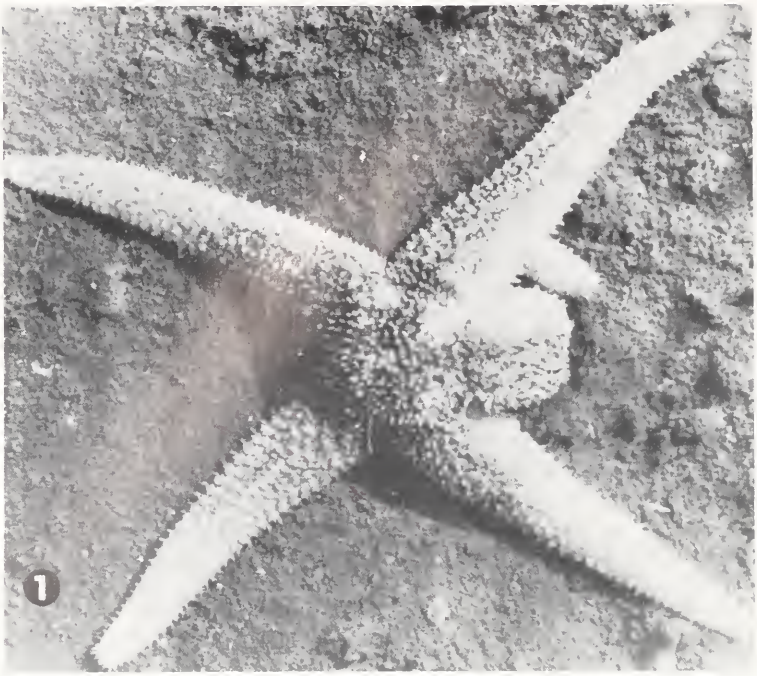
Feeding

In laboratory aquaria *L. tenera* behaved as a "sit and wait" predator. Individuals remained stationary for hours and captured small crustaceans that contacted their arms. Prey were grasped both by pedicellariae and tube feet on a seastar's arm and transferred to the mouth by flexing the arm under the disc (Fig. 1). Laboratory and field observations showed that when particularly large food items were ingested only the portion of an object within the pyloric stomach was digested. Generally, only one or two items were found in the stomachs of actively feeding *L. tenera*

TABLE II

Nearest-neighbor analysis of the spatial distribution of Leptasterias tenera *in Block Island Sound based on random underwater photographs. Calculation of the index of dispersion and statistical significance is described in the text.*

Photograph number	R	Z	Significance
STATION C 5 VIII 1970			
1	1.036	0.294	NS
2	0.892	−1.000	NS
3	0.589	−2.954	0.01
4	0.693	−2.613	0.01
5	0.545	−5.000	0.01
STATION C 9 XII 1971			
6	0.820	−1.395	NS
7	0.657	−2.064	0.05
8	1.006	0.045	NS
9	0.865	−1.364	NS
10	1.095	0.447	NS
11	0.962	−0.272	NS
12	1.174	0.669	NS
13	0.669	−2.159	0.05
STATION D 9 XII 1971			
14	1.036	0.321	NS
15	0.868	−0.790	NS
16	0.948	−0.464	NS
17	0.994	−0.045	NS
18	1.101	0.061	NS
19	0.914	−0.614	NS
20	0.864	−1.053	NS



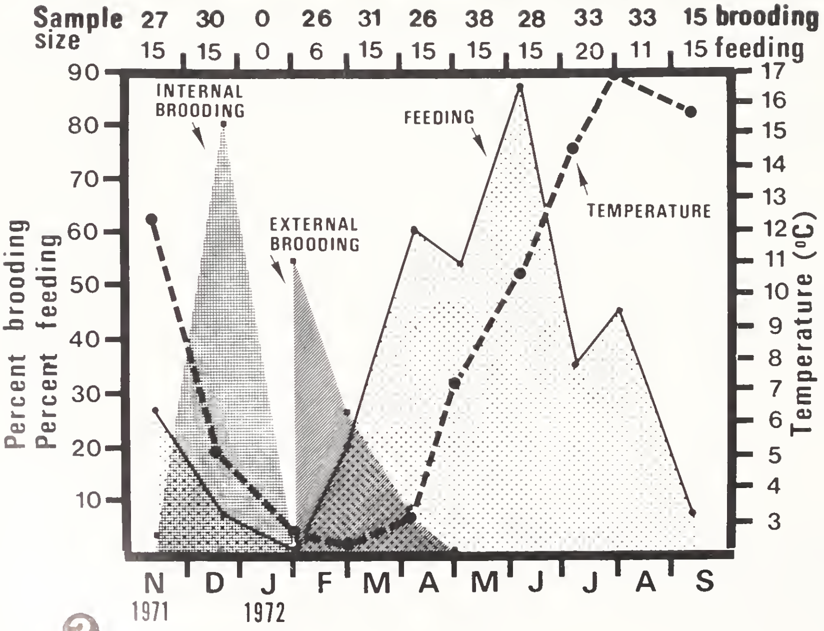


FIGURE 1. *Leptasterias tenera* feeding on an amphipod in a laboratory aquarium. The prey was captured with pedicellariae on the dorsal surface of the bent arm, transferred to the tube feet, relayed proximally along the arm, and brought to the mouth by arm flexure.

FIGURE 2. A composite graph showing the percentage of *Leptasterias tenera* with prey in their stomachs, the percentage of seastars brooding embryos in their pyloric stomachs (internal) and in an

taken in field collections although up to five amphipods were found in the stomach of a single specimen.

The percentage of feeding *L. tenera* in the population varied seasonally (Fig. 2). Feeding peaked in June, declined during the summer, and was lowest in winter. While feeding rates approached zero in February, our sample size in this month was too small to rule out the possibility that *L. tenera* feeds during this period. In a supplementary sample collected on 29 February 1972, 5 of 25 males (20%) contained partially digested amphipod remains while 20 males (80%) had empty stomachs. Of the 5 females, 3 had empty stomachs.

The benthic community at the sampling sites in Block Island Sound was dominated by amphipods, with *Ampelisca agassizi* (Judd) making up 90% of all individuals. In spite of high amphipod numbers, the community was rich in polychaete species (25 of a total of 41), only a few of which were abundant (Table III). Stomach content analysis of 177 *L. tenera* collected between November 1971 and December 1972 indicated that *A. agassizi* made up 95% of their prey items (Table IV). Despite the diversity and numbers of polychaetes available, few were found in seastar stomachs.

In northeastern Block Island Sound near Fishers Island, the bottom sediments were coarse sand and gravel which supported a high diversity of epibenthic species (Abbott, 1971) but lacked the carpets of ampeliscid amphipod tubes which occurred elsewhere in Block Island Sound. Here *L. tenera* fed on a greater variety of prey species, and relatively few amphipods (Table IV).

Seasonality and reproduction

Female *L. tenera* in Block Island Sound spawned and began to brood in November and continued to brood through March (Fig. 2). The few seastars still holding young at the beginning of April were brooding slow-developing, abortive embryos. Thus, the period of brood protection encompassed approximately four months, although each female did not necessarily brood over that entire period.

The gonad index of *L. tenera* decreased abruptly during winter spawning (from November to December) then gradually increased beginning in June the following year (Fig. 3). The pyloric caecum index reached a maximum value during the extensive spring–summer feeding period with the highest monthly mean in June. It dropped during the period of maximum gonadal growth (as the feeding rate fell), showed a significant decline by the beginning of August, and slowly decreased during the winter. The general trends in organ indices were similar for males and females, although the male gonad index increased more precipitously and reached a significantly greater value than the ovarian index.

In March, free-living juvenile *L. tenera* appeared in the benthic samples in Block Island Sound (Fig. 4), indicating that the brooding period was ending. There was some evidence of regular winter brooding for other populations of *L. tenera*. We examined specimens of *L. tenera* from the Cape Cod Bay Faunal Survey (Young, *et al.*, 1971). The gonad lengths measured as a percentage of total arm length for seastars from samples taken over a two year period showed a sharp decrease in gonadal size by December 1967 and January 1968, suggesting that

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external brood chamber, and the bottom water temperature. The number of specimens examined in the monthly samples for feeding and reproduction is indicated at the top of the graph. The samples were collected (1971) 1 October, 12 November, 21 December, (1972) 1 February, 29 February, 7 April, 2 May, 8 June, 6 July, 2 August, and 11 September.

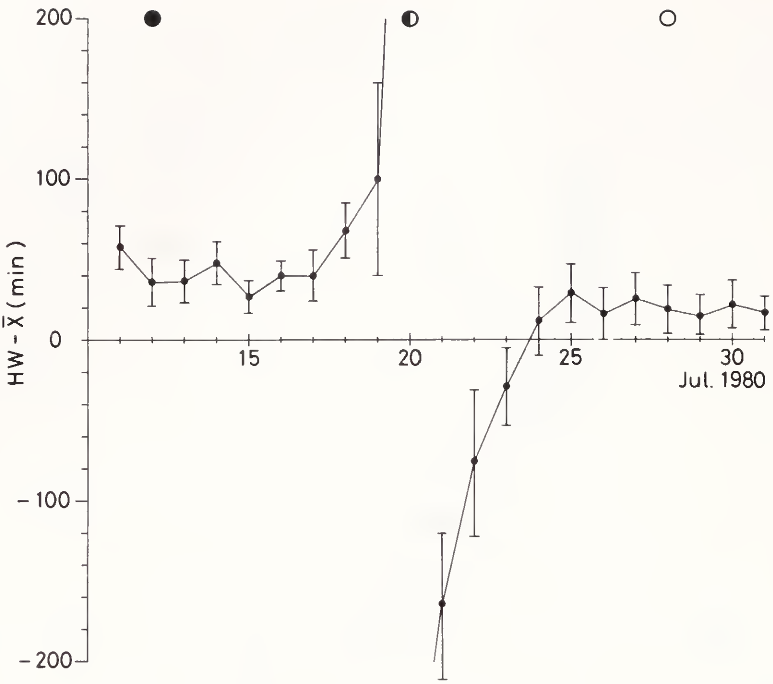

FIGURE 6(A): Correlation between the timing of larval release by the Kasaoka population and the time of high water, 11–31 July 1980. The estimated time from the average peak of the larval release activity ($\bar{X}$) to the time of high water (HW) is plotted. *HW* indicates the time of high water with which larval release coincided. Temporal variation in larval release activity is indicated by standard deviation. Though a nightime period virtually extended two days, larval release from 24:00 till dawn is included on the date preceeding the actual date.

of the Izu Peninsula. Thus, high water in Kasaoka occurs about noon and midnight at the spring tides and around sunrise and sunset at the neap tides.

In this study site, the river flat emerged at the low water periods, when the river became 5–6 m wider and about 5 cm deeper. The entire estuarine flat was submerged during the high water periods, when the maximum depth of the middle part of the river was recorded at night to be 2.5 m in July and 2.2 m in September. Although few measurements were made of the daytime high water, the tide table indicated that the water level during summer was always lower at the daytime high water than at the nighttime high water, and that the difference between them was 30–50 cm at the spring tides. The water level at the neap tides was recorded at 1.5–1.8 m in the morning and evening high-water periods. Though some difference could be seen in the water level at the high water periods according to the season and lunar phase, it had relatively little influence on the period of emersion and submersion of the mud flat (Fig. 3).

Salinity changed remarkably with ebb and flow of the tides. Also, at high water periods, a considerable difference in salinity was often recorded between water edge or surface and a place where the water was somewhat deeper. The periodic fluctuations in salinity showed a similar pattern in spring and neap tides (Fig. 4).

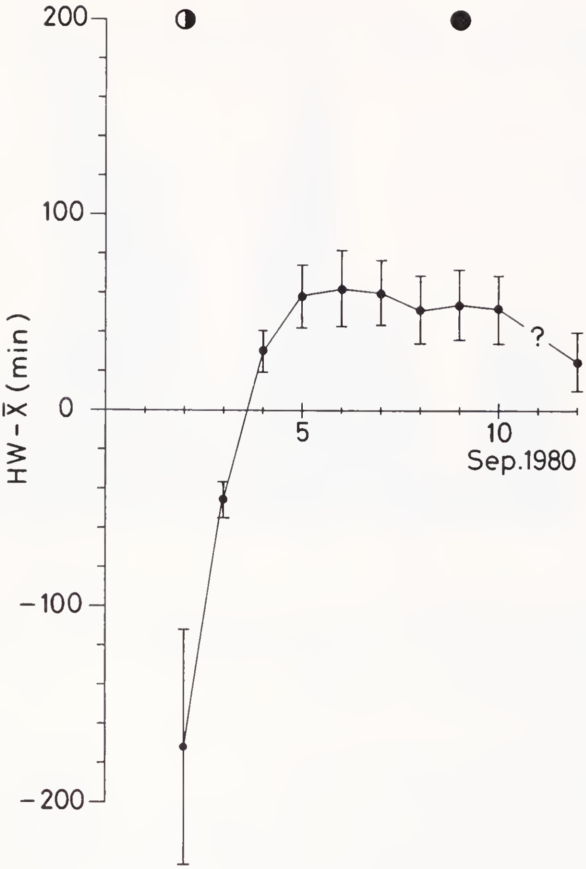

FIGURE 6(B): Correlation between the timing of larval release by the Kasaoka population and the time of high water, 1–12 September 1980. The value for HW-X̄ was calculated by the same method shown in Figure 6A.

Semilunar rhythm of larval release at Kasaoka

The time of day of larval release at Kasaoka is shown in Figure 5. It is evident that the time of day of larval release did not coincide with a lunar day cycle, but with a local tidal cycle. Larvae were released only at night, suggesting that the solar day cycle suppressed daytime larval release in all cases. In addition, larval release activity revealed a combined pattern of 24 h solar day and 24.8 h unimodal tidal components, corresponding to the phase relationship between a solar day cycle and 12.4 h tidal cycle. The solar day component appeared when high water came about sunset and sunrise, and the tidal component appeared at different times.

When the larval release activity revealed a strong correlation with nighttime high water, the average activity peak (X̄) was a little before the high water (HW) (Fig. 6A, B). The estimated time from X̄ to HW then differed somewhat in tidal cycles during the night: 42 min from 11 to 17 July, 17 min from 24 to 31 July, and 49 min from 4 to 12 September, respectively on the average. The average peak

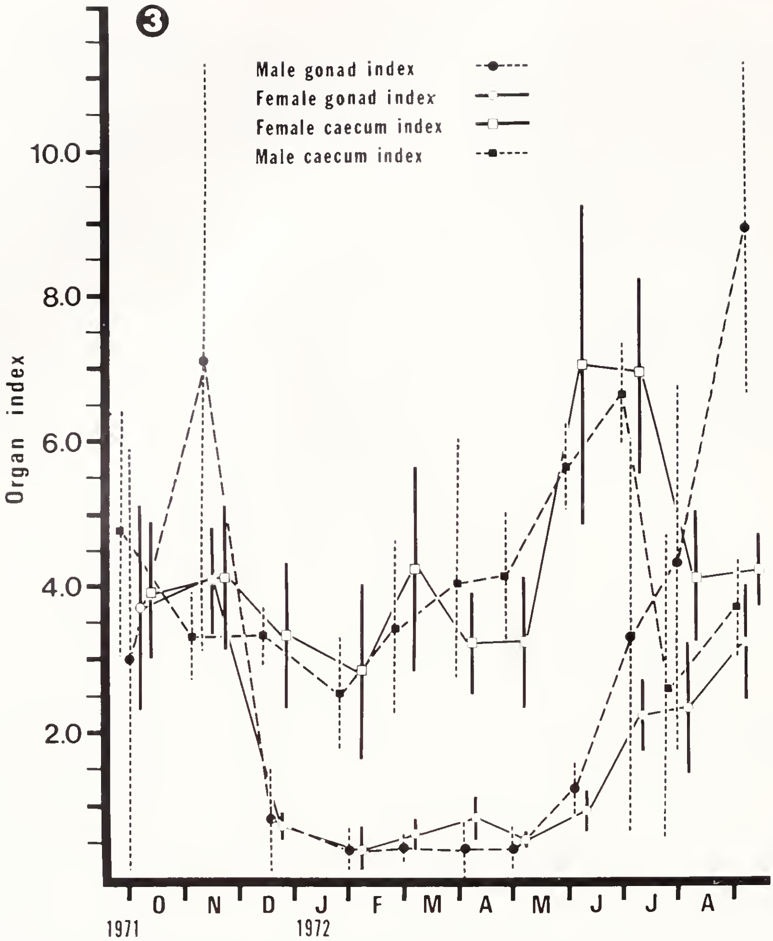

FIGURE 3. Organ indices for *Leptasterias tenera* in Block Island Sound. The mean and 95% confidence intervals are shown for the data from 5 males and 5 females in each sample. Sampling dates as in Figure 2.

that the young seastars showed a 1:1 sex ratio, but that the sex ratio for individuals of breeding size differed significantly from unity, favoring females.

Fecundity and gonadal dimorphism

L. tenera examined typically had two gonads in each arm. The ripe ovaries were small, lobate, and concentrated at the base of the arm, and mature testes were branched and some (by folding) exceeded the arm in length (Worley, *et al.*, 1977). The contrasting size of the gonads in males and females of different sizes is shown in Figure 8. The length of the ovaries was directly proportional to the size of the seastar (Fig. 8). The number of oocytes was also a direct function of the size of the female (Worley, *et al.*, 1977).

Coefficients of determination (r^2) calculated from the sets of data for males and females show that 91% of the variability of ovary length, but only 18% of the variability of testis length, is explained by the size of the seastar (Fig. 8). Thus, for male and female seastars of identical size, there is less variability expected in ovary length than in the length of the testes. In addition, the testes were often degenerated or even absent in some arms. During the period of maximum gonadal development, an average of only half of the testes in mature males were fully developed. Of the rest, 25% were drastically less than the maximum size and darkly pigmented, and some rays lacked one or both testes (n = 29 males, from combined samples collected 12 November 1971 and 11 September 1972). Female *L. tenera* occasionally had less than 10 ovaries but such morphological irregularities were considerably rarer than in males (one individual with only 8 ovaries was found among 330 dissected females).

DISCUSSION

Feeding

The feeding habits of *L. tenera* differ from those of the intertidal species of the Pacific Coast of the United States including *L. aequalis, L. hexactis,* and *L. pusilla* (Smith, 1971; Menge, 1972). The habit of prey capture with pedicellariae, and internal digestion rather than stomach eversion, restricts the prey of *L. tenera* to small organisms (primarily small crustaceans and mollusks). It is interesting that *L. littoralis* (Stimpson), a 5 armed, northwestern Atlantic species, has a diet similar to *L. tenera* and ingests small prey using its tube feet for prey capture (O'Brien, 1976). The only other seastars known to capture prey with pedicellariae are *Stylasterias forreri* (de Loriol) and possibly *Labidiaster annulatus* Sladen (Robilliard, 1971; Chia and Amerongen, 1975; Dearborn, 1977). Unlike *Stylasterias, Leptasterias* does not have greatly modified pedicellariae (Fisher, 1930). This supports Robilliard's (1971) contention that pedicellaria capture is a modification of a generalized asteroid behavior pattern that is not confined to morphologically specialized forms.

L. tenera, as well as *L. hexactis, L. pusilla,* and *L. littoralis*, exhibits an annual unimodal feeding pattern (Smith, 1971; Niesen, 1973; O'Brien, 1976). The incidence of feeding individuals in the population peaks in the late spring to early summer and declines before the occurrence of the annual maximum bottom temperature (Fig. 2).

Our data suggest that, in central Block Island Sound, *L. tenera* occur more or less randomly within a prey patch of *Ampelisca* tubes. In bottom photographs much of the area covered by amphipod tubes appears homogeneous, although some aggregations of seastars occur within this habitat (Table II). However, coarse-scale patchiness of the benthic habitat is related to areas of shelly bottoms, notably shells of *Arctica islandica*, which appear as windrows in bottom photographs, and which lack both amphipod tubes and *Leptasterias*.

Amphipods dominate the macrobenthos in the central basin of Block Island Sound (Table III) and are also the dominant taxa in the diet of *L. tenera* (Table IV). In Cape Cod Bay, *L. tenera* feeds extensively on caprellid amphipods (O'Brien, pers. comm.). However, in northern Block Island Sound, amphipods are less abundant and the diversity of prey taxa in the diet of *L. tenera* increases, probably in correlation with the composition of the benthic community (Table IV). Although amphipods are the major component of the diet in central Block Island Sound, there is no evidence from this study that *L. tenera* feeds preferentially on amphipods. However, O'Brian (1976) found that in simple choice experiments, amphipods were the preferred prey of *L. littoralis*. There is a possibility that *L. tenera* may also prefer amphipods, but experimental evidence is needed.

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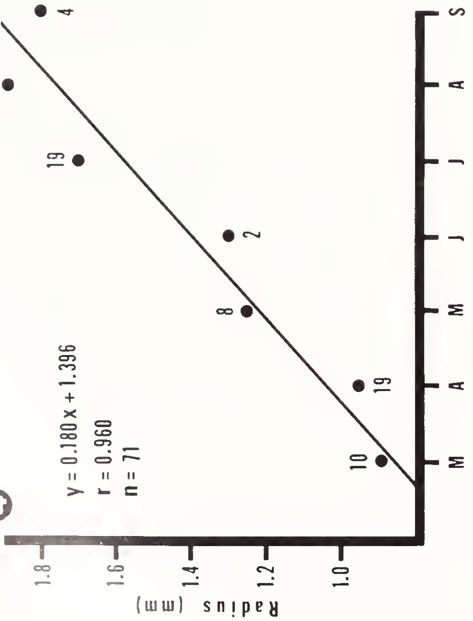
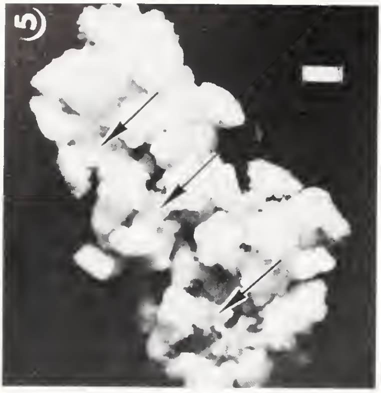

Radius (mm)
1.8
1.6
1.4
1.2
1.0
M
A
M
J
J
A
S
y = 0.180 x + 1.396
r = 0.960
n = 71
10
19
8
2
19
9
4

⑤



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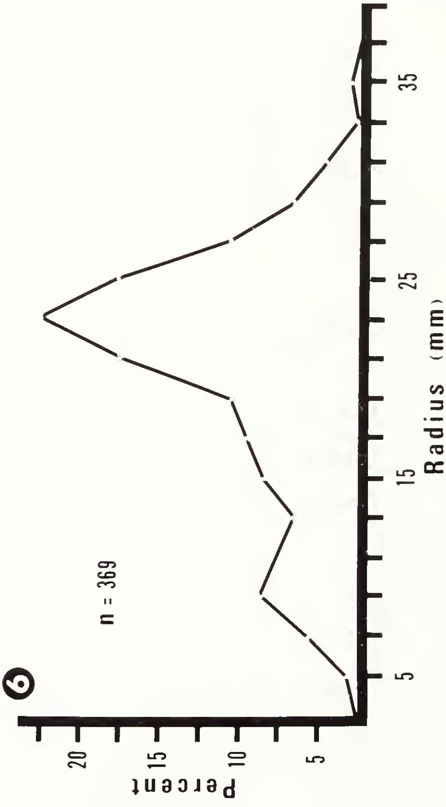
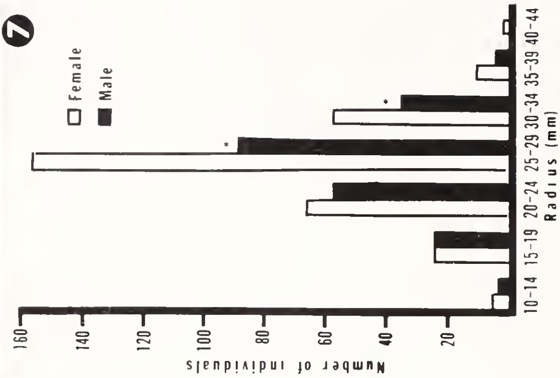

Percent
20
15
10
5
n = 369
5
15
25
35
Radius (mm)

⑦


Number of individuals
160
140
120
100
80
60
40
20
☐ Female
■ Male
*
*
10-14
15-19
20-24
25-29
30-34
35-39
40-44
Radius (mm)

Polychaetes do not appear to make a significant contribution to the diet of *L. tenera* even though the central basin of Block Island Sound supports a relatively diverse worm fauna (Table III). We do not know whether *L. tenera* avoids polychaetes or is simply unsuccessful at capturing them. However, other holophagous asteroids, notably species of *Astropecten*, discriminate against polychaetes (Christensen, 1970; Ribi, *et al.*, 1977; Franz and Worley, pers. comm.).

Other *Leptasterias* species also have circumscribed diets and a seeming tendency to prey on locally abundant resources. For example, subtidal populations of *L. hexactis* in Puget Sound, Washington, feed almost exclusively on the holothuroid *Cucumaria lubrica* Clark, and in kelp communities feed on the gastropod mollusk *Lacuna* spp. (Mauzey, *et al.*, 1968; Niesen, 1973). Feder and Jewett (1980) report that in the southeastern Bering Sea, *Leptasterias polaris acervata* (Stimpson) feeds solely on the cockle, *Clinoncardium ciliatum* (Fabricius). However, it is noteworthy that sympatric *Leptasterias* species may show different feeding habits. *L. pusilla*, in California tide pools, feeds almost entirely on the small snail *Barleeia acuta* (Carpenter), while *L. aequalis* in the same habitat consumes gastropods, mussels, limpets, polychaetes, and even small seastars and detritus (Smith, 1971).

Modes of brooding in Leptasterias *species*

Three modes of brooding have been described in previous studies of *Leptasterias*. In the first type, exhibited by *L. ochotensis similispinis* (Clark), the eggs adhere to the substratum beneath the female (Kubo, 1951). In the second type (*e.g.* in *L. aequalis* (Stimpson), *L. alaskensis* (Verrill), *L. hexactis* (Stimpson), *L. muelleri* (Sars), and *L. pusilla* Fisher) the female holds the eggs and embryos in a "brood chamber" beneath her arched disc and arms (Sars, 1846; Fisher, 1930). In the third mode, that of *L. groenlandica* (Lütken), the female holds both the eggs and embryos in the cardiac portion of her stomach until the juveniles emerge (Lieberkind, 1920).

L. tenera shows characteristics of both the second and third modes described above. Embryos of *L. tenera* (at least until the late gastrula stage) are held in the pyloric chamber of the mother's stomach, and brachiolariae and later stages reside in a brood chamber beneath the disc of the female before they emerge and disperse. The behavioral mechanism involved in embryo transfer was not observed, but it might resemble that described by Chia (1966) for *L. hexactis*.

Our examination of the literature shows that *Leptasterias* species with external and internal brooding have different types of embryos. Those species which brood internally have an amorphous membranaceous nucleus of unknown composition to which the embryos attach (Fig. 5). The embryos of *L. groenlandica* are attached to such a central mass by a long, thin preoral lobe that is tipped with minute brachiolar arms (Fisher, 1930), as are the embryos of *L. tenera*. It is possible that

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FIGURE 4. A plot of the mean size of juvenile *Leptasterias tenera* from semimonthly collections in Block Island Sound, October 1971 to May 1972. Sample size is indicated by the number near each datum point.

FIGURE 5. A cluster of *Leptasterias tenera* embryos photographed on 1 February 1972. At this stage the brachiolar appendages (arrows) which connect the embryos to a central membranaceous mass are capable of moving, as are the tube feet of the developing seastars. Scale is 1 mm.

FIGURE 6. Size-frequency distribution of adult *Leptasterias tenera* dredged between Stations C and D, west of Block Island, 9 July 1974.

FIGURE 7. Size-frequency histograms of male and female *Leptasterias tenera* collected in Block Island Sound from October 1971 to September 1972. Each size class was tested for agreement with a 1:1 sex ratio, and size classes differing significantly from unity (Chi-squared test, $P < 0.05$) are marked with an asterisk. No males were sampled in the 40–44 mm size group.

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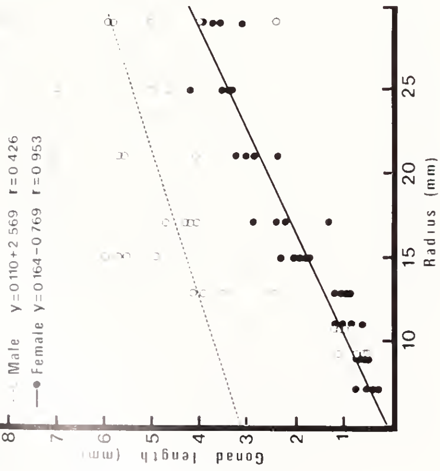
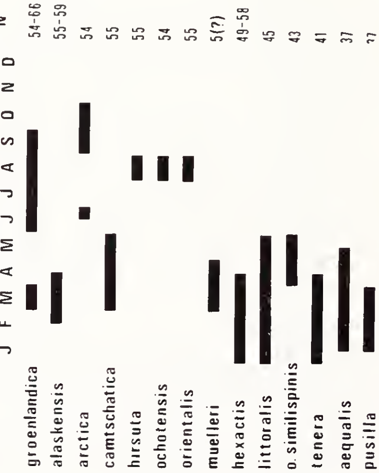
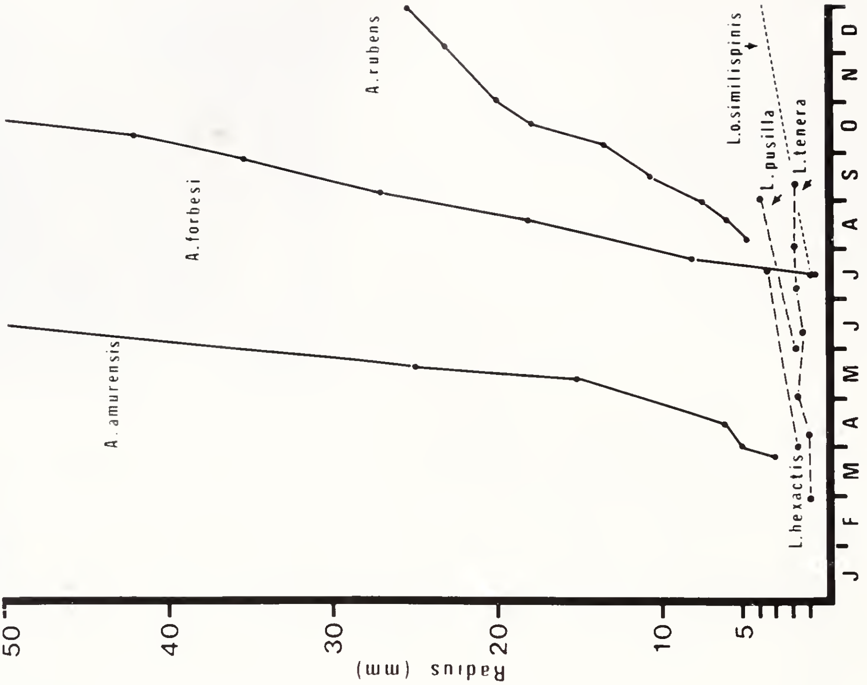

Male y=0.110+2.569 r=0.426
Female y=0.164−0.769 r=0.953
11.4
10
Gonad length (mm)
8
7
6
5
4
3
2
1
10
15
20
25
Radius (mm)

9


Brooding period
J F M A M J J A S O N D
Lat. °N
groenlandica
54-66
alaskensis
55-59
arctica
54
camtschatica
55
hirsuta
55
ochotensis
54
orientalis
55
muelleri
5(?)
hexactis
49-58
littoralis
45
o. similispinis
43
tenera
41
aequalis
37
pusilla
?7

10


A. amurensis
A. forbesi
A. rubens
L. o. similispinis
L. pusilla
L. tenera
L. hexactis
50
40
30
20
10
5
Radius (mm)
J F M A M J J A S O N D

the central mass is composed of fertilization membranes which coadhere after the embryos hatch in the mother's stomach.

The embryos of externally brooding species have compact preoral lobes and do not develop bonds to each other or to the brood chamber (*e.g.* Gordon, 1929; Chia, 1968a). As a test of this inferred correlation between the mode of brooding and the morphology of the embryos we predict that *L. arctica* (Murdoch), which Fisher (1930) illustrated with a nucleus of embryo attachment, broods embryos within its stomach.

Although there may be phylogenetic affinities between species with similar brooding types (*e.g.*, all known purely external brooders are in the subgenus *Hexasterias*, the 6 armed *Leptasterias*), it is possible that brooding specializations have evolved in response to the specific environmental pressures experienced by each species. *L. ochotensis similispinis*, for example, spawns on hard substrates. Its egg masses are sessile and the female remains stationary during brooding (Kano, *et al.*, 1974). In contrast, internal brooding may permit greater freedom of movement, an adaptation especially important for species such as *L. tenera* living on soft or unstable substrates. A centrally connected embryo mass could facilitate retention of a brood by a moving female. On the other hand, for species occupying hard substrates in high energy environments, unconnected embryos could be advantageous since less than the whole clutch need be lost if a brooding female were dislodged by wave action. Finally, external brooding may raise the limits of brood size for some species, since the clutch volume of internal brooders must be limited by the capacity of the stomach to accommodate and maintain viable ova.

Cycles of reproduction

L. tenera in Block Island Sound, Cape Cod Bay, and Massachusetts Bay spawns during the winter (Fewkes, 1888; this paper). This reproductive pattern may be related to the natural decrease in prey abundance during cold weather (Chia, 1968b), or to the seasonal inactivity of predators on seastars and their embryos. The spawning of *L. hexactis* and *L. pusilla* during the winter could help provide the newly released young with an optimal supply of food (Chia, 1968b; Smith, 1971; Menge, 1975). However, apart from Niesen's (1973) observations that young *L. hexactis* feed on newly settled *Spirorbis* species, there is no information on the diet of juvenile *Leptasterias* species in the field.

The literature indicates that many *Leptasterias* species brood during the winter. Exceptions are, for example, *L. ochotensis similispinis* in Japan which broods during April–May, and the brooding specimens of high latitude species collected

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FIGURE 8. Gonad length as a function of body size (radius) of male and female *Leptasterias tenera*. Each point represents the mean length of the gonads in one seastar.

FIGURE 9. Brooding periods of *Leptasterias* species arrayed according to latitude, showing that the brooding period is shorter in a low than in high latitudes. Data on the duration of brooding periods are incomplete for species north of 50°N latitude. References: *L. groenlandica*, Djakonov, 1938; Fisher, 1930. *L. camtschatica*, Djakonov, 1938. *L. hirsuta*, Djakonov, 1938. *L. ochotensis*, Djakonov, 1938. *L. orientalis*, Djakonov, 1938. *L. muelleri*, Sars, 1846; Mortensen, 1927. *L. hexactis*, Osterud, 1918; Menge, 1974. *L. littoralis*, Verrill, 1895. *L. ochotensis similispinis*, Kubo, 1951. *L. tenera*, this report. *L. aequalis*, Fisher, 1930; Hewatt, 1938; Smith, 1971.

FIGURE 10. Growth rates of newly settled *Leptasterias* and co-occurring forcipulate seastars. References: *L. hexactis*, Osterud, 1918. *L. pusilla*, Smith, 1971. *L. ochotensis similispinis*, Kano, *et al.*, 1974. *L. tenera*, this report. *Asterias amurensis*, Ino, *et al.*, 1955; Hatanaka and Kosaka, 1959. *A. forbesi*, Mead, 1900. *A. rubens*, Barnes and Powell, 1951. The growth rate of *L. ochotensis similispinis* is based on an extrapolation between measurements of the mean size of individuals in the first growth season and of the first year class.

during the summer months (often the only season when the northernmost taxa are accessible) (Fig. 9). The relationship between latitude and brooding period in the genus as a whole (Fig. 9) seems to fit the pattern expected for a high latitude species, with a brief spawning period during low temperature seasons in warm climates and more extended spawnings in cold climates (Orton, 1920). As more data accumulate on the reproductive periodicity of *Leptasterias* species it may be possible to determine whether the distribution of the genus is limited to regions with water temperatures required for successful reproduction. For certain species temperature can clearly be a critical factor in reproduction, and O'Brien (1976) has indicated that falling temperatures may trigger spawning in *L. littoralis.* Although brooding does appear to be correlated with temperature, the ultimate selective factor for the brooding period is not known.

Figure 3 shows that the caecum and gonad index cycles of *L. tenera* are out of phase. This is not unexpected, for as Harrold and Pearse (1980) point out, although the inverse relationship between the gonadal and caecum indices has often been emphasized, not all seastars show a strict inverse pattern. The change in the size of asteroid caeca is probably related to multiple functions of the organs (digestion and mobilization, distribution, and storage of nutrients) and is strongly influenced by temperature sensitive rate functions and the availability of food, rather than being primarily determined by reproductive effort (Lowe and Dearborn, 1979; Van der Plas, *et al.*, 1980; Oudejans, *et al.*, 1980; Harrold and Pearse, 1980).

The caecum indices of male and female *L. tenera* are similar and show a slight (not statistically significant) decrease during the brooding period. The male gonad index, however, rises more abruptly and reaches a higher value than the gonad index of the females (Fig. 3). Some populations of *L. hexactis* and *L. pusilla* have organ index patterns much like *L. tenera* (Smith, 1971; Niesen, 1973). For example, *L. hexactis* in Oregon may show no detectable decrease in the caecum index during brooding (Niesen, 1973). These observations appear to contradict Menge's (1975) hypothesis that brooding female *L. hexactis* must invest more energy than males do in the storage of nutrients and that their caecum index should drop more sharply than the male index during the brooding period because the brooding females cannot feed and recoup nutrient losses.

Growth and population dynamics

Since the smallest individuals to develop gonadal tissue were 5 mm, gonadal development may begin in year-old seastars based on the rate of growth estimated above. Since oogenesis requires a total of 24 months (Worley, *et al.*, 1977) and mature oocytes appear for the first time in animals no smaller than 15 mm, these sexually mature animals must be about 3 years of age (in their fourth growing season). Therefore, it is likely that the first modal group of seastars (the 9 mm group in Fig. 6) is in its third growing season and the seastars of 15–30 mm (the bulk of the population) and a small number of much larger specimens, may comprise older year classes (Fig. 6). Thus, on the basis of the population size-frequency distribution, we suggest that *L. tenera* lives 4 or more years. Since ripe oocytes are already present in females as small as 15 mm radius (about 3 years old) and gonads are found in individuals as large as 40 mm, *L. tenera* is probably iteroparous.

The growth rate of *L. tenera* is slow compared with broadcasting seastars such as *Asterias* species, but similar to other *Leptasterias* species (Fig. 10). Even *Pisaster ochraceus* (Brandt), a slow-growing broadcast spawner achieves a greater growth rate than *Leptasterias* species, as it attains 22 mm radius in one year (Quayle, 1954).

Menge (1975, p. 96) offers an ". . . interpretive hypothesis that brooding in *L. hexactis* is an evolutionary response to competition-induced small body size." Specifically, he suggests that brooding in *L. hexactis* is a "coadaptive consequence" of competition with *Pisaster ochraceus*. However, the genus *Leptasterias* is largely composed of cold water (arctic–boreal), slow-growing species (Figs. 9, 10). Thus, it is possible that brooding and associated specializatons such as a two-year period of oogenesis (Chia, 1968a, b; Worley, *et al.*, 1977) and a slow rate of growth (Fig. 10) are widespread "cold adaptations" (*sensu* Clarke, 1980) shown by the genus *Leptasterias* rather than the evolutionary consequence of competition in certain species.

There is no evidence that female *L. tenera* suffer greater mortality than males as a consequence of brooding. As inferred from Fig. 7, male morality exceeds female mortality for sexually mature individuals in the Block Island Sound population. While the behavioral activities associated with spawning may extract some metabolic costs, these have not been measured, nor has the degree and importance of starvation during brooding.

In the winter, we found that feeding activity appeared to be depressed in both male and female *L. tenera*, though our data were few. O'Brien (1976) has suggested that brooding female *L. littoralis* may feed on fine particulate material while brooding. The uptake of dissolved organic materials is also possible, and (though rarely) brooding female *L. hexactis*, *L. pusilla*, and *L. tenera* have been found with ingested prey (Osterud, 1918; Smith, 1971; Menge, 1974; pers. obs.). Moreover, the histological changes which have been interpreted to suggest that female *L. hexactis* cannot feed while brooding are also found in non-brooding species that are capable of feeding throughout the year (Chia, 1969; Nimitz, 1971). Thus, the possible significance of nutrient uptake by brooding female *Leptasterias* invites additional experimentation.

The pyloric caecum index has been used to estimate the energy expenditure involved in brooding (Menge, 1974). However, gametogenesis and maintenance metabolism also draw upon the seastar's energy reserves during the brooding period (Chia, 1968b; Worley, *et al.*, 1977). As Harrold and Pearse (1980) and Lowe and Dearborn (1979) have shown, the allocation of energy from the pyloric caeca is not well enough understood to assume (as suggested by Menge, 1974) that the change in the caecum index before and after brooding approximates the weight an individual loses to gametes and brood handling. Clearly, additional data are required to assess reliably the energetic costs of brooding.

A first approximation of the relative costs of brooding versus broadcast spawning might be developed by comparing the weight of spawned gametes in representative species. The brooding species *L. hexactis* and *L. tenera* lose an estimated 5.5% (Menge, 1974) and 5.6% (this study) of their total body weight during spawning, as compared to from 7–10% and up to 40% in the broadcast spawner *Pisaster ochraceus* (Menge, 1974). We therefore suggest that until accurate calorific budgets are available it should not be assumed that the brooding *Leptasterias* species allocate more energy to reproduction than do broadcast spawning asteroids.

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THE FEEDING RESPONSE OF *HYDRA VIRIDIS*: EFFECTS OF PREY DENSITY ON CAPTURE RATES

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ABSTRACT

A model of the feeding processes of *Hydra viridis* was developed and used to predict the environmental parameters which maximize feeding.

Feeding was measured by exposing individual hydra to *Artemia salina* nauplii and recording the number ingested. When fed to repletion *H. viridis* resumed feeding after 4.8 hours and ingested significantly fewer *Artemia* during the second feeding. At low prey densities, increasing the exposure time from 15 to 60 minutes increased the number of *Artemia* ingested. However, at higher densities exposure time did not affect the number of *Artemia* ingested. There was a strong correlation between the number of *Artemia* captured and the number ingested. Increased prey capture did not alter the duration of the feeding response but did reduce the time interval between ingestions. Exposure to *Artemia* extract reduced the number of *Artemia* ingested and the duration of the feeding response.

These data indicate that feeding success under conditions of low prey density is limited by the availability of prey. At high prey density the feeding process is itself saturated and prey availability has limited effects on ingestion. *H. viridis* is well suited to high density feeding and ingests more prey when prey density is high even if the total exposure to prey is maintained at a constant level.

INTRODUCTION

The manner in which an organism extracts nutrients from its surroundings, feeding, is one of the organism's most basic interactions with the environment. Like other phenotypic characters, feeding can be studied as an adaptation of a species to its environment. Feeding is, however, a function of many parameters and an integrative study can illustrate the manner in which physiological, behavioral, and morphologic characters co-evolve and lead to successful feeding in a given environmental millieu.

Hydra are particularly well suited for a detailed study of feeding since extensive work has already been carried out. Laboratory and field data both indicate that feeding rates are particularly important in regulating *Hydra* population densities. Laboratory studies (Lenhoff and Loomis, 1957; Muscatine, 1961; Muscatine and Lenhoff, 1965) have repeatedly shown that *Hydra* population growth rates are directly related to the frequency of feeding. Slobodkin (1964) has even suggested that *Hydra* population size cannot exceed the number of available food particles. In a field study Cuker and Mozley (1981) showed that *Hydra* population densities closely followed increases in zooplankton abundance, and they experimentally demonstrated that increased feeding by the *Hydra*, when zooplankton were abundant, could account for the observed increase in the *Hydra* population.

The feeding process of *Hydra* has been divided into a series of discrete steps:

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the capture of prey with nematocysts, transport of prey to the mouth, mouth opening, ingestion, digestion, and egestion (Forest, 1962). The steps between the capture of a prey item and its ingestion have been termed the feeding reaction or response (Loomis, 1955; Lenhoff, 1961a) and have been examined in great detail. The response in *Hydra* is triggered by glutathione (Lenhoff, 1961a; Mariscal, 1971), and a wide variety of environmental parameters are known to affect the response (*c.f.* Lenhoff and Boviard, 1959; Lenhoff, 1961b, 1965). However, the effects of stimuli on the feeding rate, *i.e.,* the amount of food ingested, are not known.

In this paper we develop a model of the feeding process in *Hydra viridis*, and discuss how the morphology, physiology, and behavior of *H. viridis* interact to make *H. viridis* an effective feeder in aquatic environments in which prey are concentrated in dense patches.

MATERIALS AND METHODS

Cultures of *Hydra viridis* (Carolina Biological Supplies strain) were maintained in M solution (Lenhoff and Brown, 1970) at 20 ± 1°C, on a 12 hour light/dark photoperiod at a light intensity of 65 $\mu E \cdot m^{-2} \cdot sec^{-1}$. Hydra were fed every third day with freshly hatched *Artemia salina* nauplii. Hydra used in experiments were starved for two days in mass culture. Only hydra with a single bud were used in experiments. All experiments were conducted in 35 mm petri dishes containing a single hydra in 3 ml of M solution.

Satiation of feeding was examined by allowing 10 individual hydra to feed to repletion on an excess of *Artemia* nauplii. *Artemia* were then presented to the hydra every 30 min until feeding resumed. The number of *Artemia* ingested by each hydra during both feedings, as well as the time to resumption of feeding, was recorded for each hydra.

The density of prey available to *H. viridis* when fed to repletion probably exceeds natural prey densities. To examine the effects of prey density and exposure time on ingestion 10 individual hydra were exposed to 1.7, 3.3, 6.7 or 13.3 *Artemia* $\cdot ml^{-1}$ and allowed to feed for 15 or 60 minutes. The number of *Artemia* ingested was determined by counting the number of nauplii remaining in the petri dish at the conclusion of the experiment.

Effects of chemical stimulation on feeding were examined by treating hydra with *Artemia* extract. *Artemia* were ground with a mortar and pestle, filtered, and the filtrate then used as a chemical stimulus. Twenty individual hydra were treated with one drop of *Artemia* extract either for 1, 5, 10, or 30 min. Hydra were then rinsed with fresh M solution and immediately fed an excess of *Artemia.* The number of *Artemia* captured, the time required for each ingestion, the number of prey ingested, and the duration of the feeding response were recorded for each hydra.

To examine whether *H. viridis* ingests more prey when exposed to prey at high density for short periods rather than at lower prey densities for longer time periods, groups of 20 individual hydra were exposed to different combinations of prey density and exposure time and allowed to feed. In all cases prey exposure (prey density × exposure time) remained constant. Cultures were checked regularly and density maintained by replacing all non-swimming or ingested *Artemia.* The number of *Artemia* ingested was determined by counting the number remaining in the petri dish at the conclusion of the experiment.

RESULTS

Observations made during the experiments are in close agreement with previous descriptions of the feeding sequence in hydra (Loomis, 1955; Lenhoff 1961a).

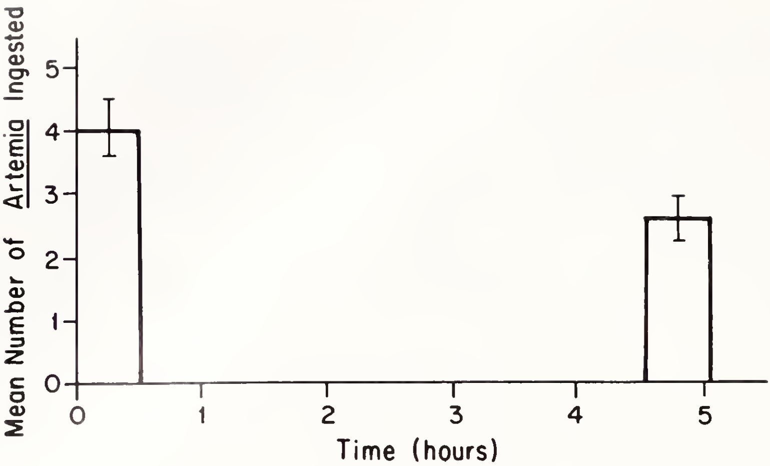

FIGURE 1. Mean number of *Artemia* nauplii ingested/hydra of 10 individual *H. viridis* fed to repletion. Hydra were offered *Artemia* every 30 min after the initial feeding until feeding resumed. Bars denote standard errors.

Nauplii, which swam about in a seemingly random fashion, would strike a single tentacle which usually resulted in the capture of a nauplius. The captured nauplius was then transported to the mouth and ingested. After a period of time (always less than 30 minutes) captured nauplii not yet ingested fell off the tentacles. Nauplii striking the tentacles at this time were sometimes captured but never ingested.

The effects of the feeding sequence on the number of prey ingested are illustrated in Figure 1, which presents the results of the satiation feeding experiments. During their initial exposure to prey *H. viridis* captured an average of 4.0 *Artemia*, but individual hydra ingested significantly different numbers of *Artemia* ($P < 0.01$, analysis of variance [ANOVA]; $F = 16.9$; df = 1, 9). Subsequent exposures to prey did not elicit any ingestions until at least 3.5 h had elapsed at which time a second feeding bout occurred. The length of time between feeding bouts was independent of the number of prey ingested during the first feeding bout ($r = 0.25$, $P < 0.05$). The average time between feeding bouts was 4.8 h. Feeding during the first feeding bout was greater than during the second ($P < 0.01$, ANOVA).

Both the density of *Artemia* nauplii and the length of exposure to prey affected ingestion rates (Fig. 2). Increasing *Artemia* density increased the number of prey ingested (ANOVA, $F = 21.7$; df = 3, 72; $P = 0.001$). Increased exposure time also enhanced the number of prey ingested (ANOVA, $F = 8.35$; df = 1, 72; $P = 0.005$). *A posteriori* *t*-tests indicate, however, that increased exposure time did not significantly affect ingestions at the high densities. This suggests that at high prey density *H. viridis* captured all of its prey within 15 min.

A more detailed analysis of feeding can be developed from an examination of the detailed observations of hydra fed to repletion (Table I). There was considerable variability in all components of the feeding sequence in these experiments. These data reveal that hydra regularly captured more prey than were actually ingested. There is, however, a positive correlation between the number of *Artemia* captured and the number ingested ($r = 0.87$, $P < 0.001$). Regression analysis indicates, however, that as the number of prey captured increased, percent prey captured actually decreased. (The slope of the regression [0.49] is less than 1.0 [$t = 4.45$, $P < 0.001$].) *Artemia* which were not ingested fell off the tentacles after the last ingestion.

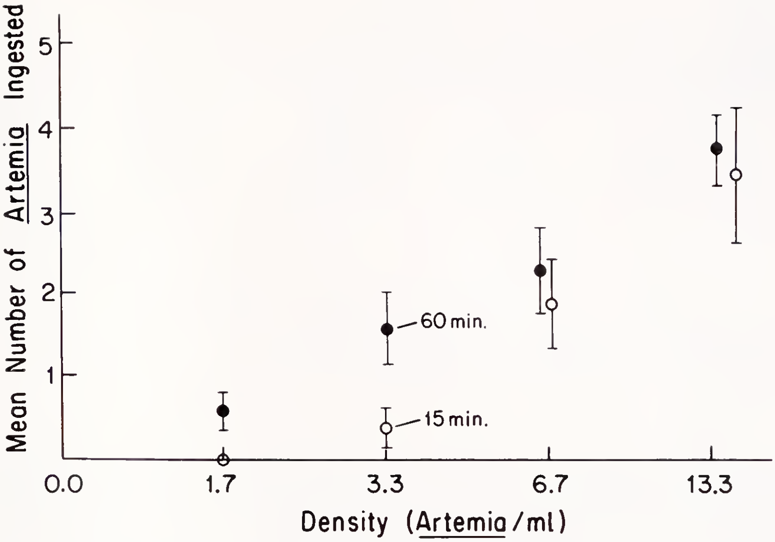

FIGURE 2. Effects of prey density and exposure time on the mean number of *Artemia* ingested by 10 individual *H. viridis* polyps fed for 15 min (○) or 60 min (●). Bars denote standard errors.

TABLE I

Results of feeding an excess of Artemia *nauplii to individual* Hydra viridis.

			TIME INTO EXPERIMENT (MIN)		
	Number of Artemia Captured	Number of Artemia Ingested	First Ingestion	Second Ingestion	End of Feeding
	3	2	6	30	30
	2	2	4	19	19
	3	2	6	15	15
	2	2	9	19	19
	3	3	3	21	21
	4	2	6	21	21
	5	3	5	12	24
	4	2	3	20	20
	4	3	7	11	26
	8	3	5	10	30
	6	2	17	30	30
	10	4	8	16	22
	8	5	6	15	23
	7	3	10	13	15
	10	7	6	9	20
	11	6	5	8	26
	14	7	4	5	28
	9	6	3	10	17
	8	4	2	15	15
	6	4	3	5	14
Mean	6.4	3.6	5.9	15.2	21.8
Standard Error	0.7	0.4	0.8	1.6	1.2

TABLE II

Effects of number of captures on H. viridis *feeding response.*

	Number of artemia captured	Number artemia ingested	Time until first ingestion (min)	Time between ingestions (min)	Length of feeding response (min)
GROUP I	3.3* (0.3)	2.3* (0.2)	5.4* (0.7)	11.6* (2.2)	21.0 (1.6)
GROUP II	8.8 (0.7)	4.6 (0.5)	6.3 (1.3)	5.6 (1.4)	21.8 (1.8)

* Group I and II differ significantly ($P < 0.005$, *t*-test).
Values are means (and standard errors) of 11 group I and 9 group II individuals.

The relationship between capture and ingestion rate in these experiments is controlled by the ingestion rate and not by the amount of time spent feeding. For analysis the data in Table I were divided in half, those hydra capturing more than five *Artemia* and those capturing five or fewer *Artemia* (Table II). Data in Table II indicate that hydra which captured larger numbers of prey spent as much time feeding as those hydra capturing lower numbers of nauplii, ($P > 0.1$, *t*-test). Hydra which captured large numbers of prey ingested nauplii at a greater rate and ingested a greater total number of nauplii ($P < 0.005$, *t*-test). Increased prey capture increased the number of prey ingested by decreasing the time interval between ingestions ($P < 0.005$, *t*-test).

The effects of *Artemia* extract on the number of prey ingested and the duration of the feeding response are depicted in Figure 3. The presence of the extract significantly reduced the duration of the feeding response ($P = 0.0016$, ANOVA, $F = 4.30$, df = 5, 88) and also reduced the number of *Artemia* ingested ($P = 0.003$, ANOVA, $F = 3.85$, df = 5, 88). The duration of the feeding response among untreated hydra was significantly greater than that of treated hydra ($P < 0.05$, least significant difference test). Treatments which differed only in the length of exposure to extract did not differ significantly from each other, but there was a significant negative correlation between duration of the feeding response and exposure to extract ($r = 0.35$, $P < 0.01$, $n = 94$). A comparison of the number of *Artemia* ingested among the treatments indicated that no single treatment differed significantly from all other treatments. However, ingestions among both untreated and 1 min treated hydra differ from the 10, 20, and 30 min treated hydra. A significant negative correlation was also observed between ingestion and exposure to extract ($r = 0.37$, $P < 0.01$, $n = 94$).

The effects of manipulating the length of time exposed to the prey and prey density are depicted in Figure 4. In each of the three experiments the total exposure to prey (*i.e.*, the probability of an *Artemia* striking a hydra) was kept constant. However, ingestion rates were significantly different ($P < 0.001$, ANOVA, $F = 18.8$, df = 2, 57) and steadily decreased with decreasing prey density ($P < 0.05$, least significant difference).

DISCUSSION

H. viridis' response to prey is modified by the feeding process itself. Therefore the response of *H. viridis* to prey cannot be modelled as a simple impact–capture–

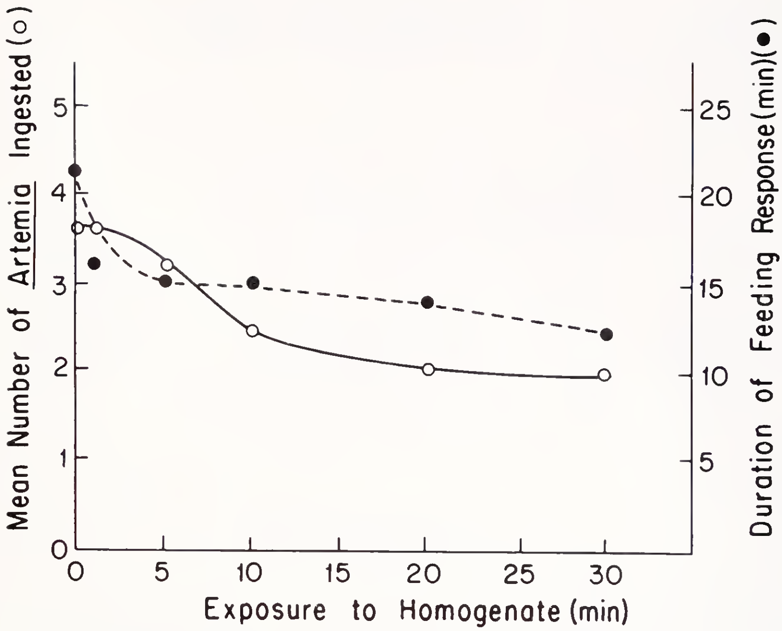

FIGURE 3. The effect of *Artemia* extract on the mean number of *Artemia* ingested (○) and duration of the feeding response (●) of 20 individual *H. viridis.*

ingestion sequence in which the probabilities of prey capture and ingestion are static. The probability of a zooplankter striking a tentacle and then being captured and ingested depends on the timing of the occurrence relative to previous feeding events. This effect is evident in Figure 5, which presents a model of feeding by *H. viridis.* The model incorporates many well known aspects of *Hydra* feeding (see Linstedt, 1971; Lenhoff, 1974) as well as the variations in feeding observed in this study.

The first step of the feeding process is the impact of a prey item with a tentacle. Tentacle movements prior to impact appear random with respect to prey movement and the rate of impacts is probably a function of prey density and prey behavior.

Following impact, nematocysts in the tentacle discharge leading to capture of the prey. Nematocyst discharge decreases when there is food in the gastrovascular cavity (Burnett *et al.*, 1960; Hand, 1961). In our experiments *H. viridis* continued to capture *Artemia* nauplii even after ingestion had ceased. However, observations of fed hydra revealed instances of nauplii swimming away after striking tentacles. Such occurrences almost never occurred at the start of feeding experiments. The ability to capture prey also appeared to decrease during the course of the 4 h digestion period.

The chemical stimulation provided by *Artemia* extract reduced the length of the feeding response (Fig. 3) suggesting that chemical stimulation of the feeding response is in some fashion time limited. However, chemical stimulation of 30 min reduced the feeding response by only 12 min which indicates that the feeding response is not controlled by chemical stimuli alone. Although the size of the gut ultimately limits the amount of food ingested, hydra frequently stopped feeding after only a single nauplius had been ingested. Thus, extension of the gut alone did not control the shutdown of the feeding response either.

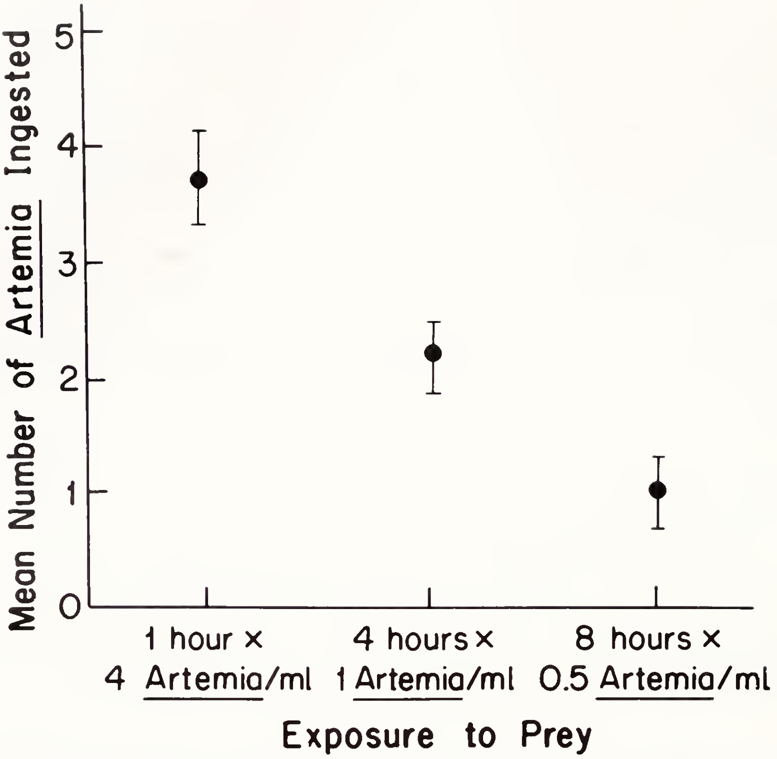

FIGURE 4. Effects of prey density and exposure time on the mean number of *Artemia* ingested/hydra by 20 individual *H. viridis* polyps. Prey exposure (*Artemia* density × exposure time) is identical in all instances. Bars denote standard errors.

Prey density affects the number of prey ingested through the ingestion rate. When large numbers of prey are captured the rate at which prey are ingested increases (Table II). Consequently, a larger number of prey are consumed during the fixed period of the feeding response.

Implicit in the model of feeding is the constraint on feeding imposed by *Hydra's* simple gut. Food must be ingested, digested, and egested in discrete batches. Consequently, *H. viridis* captures and ingests prey during short feeding bouts followed by longer periods of digestion. The time spent digesting prey limits the amount of food consumed, since prey are not captured while there is food in the gut.

The feeding sequence modelled in Figure 5 controls *H. viridis'* functional response to prey (*sensu* Holling, 1959), and the model can be used to predict the conditions in which *H. viridis* is most effective as a predator. Where prey are present in low densities, *H. viridis* are unlikely to catch and ingest more than a single zooplankter during an ingestion-digestion sequence. This limits feeding success to only a single prey every 3–5 hours regardless of the number of prey striking tentacles during the period in which ingestion is inhibited. This is evident in Figure 4 in which *H. viridis* exposed to 0.5 *Artemia* $\cdot$ ml^{-1} for 8 hours captured significantly fewer prey than *H. viridis* exposed to greater densities for less time. The number of prey consumed is lower than expected on the basis of hydra-prey encounters. Under these circumstances the feeding process exhibited by *H. viridis* appears to be particularly disadvantageous.

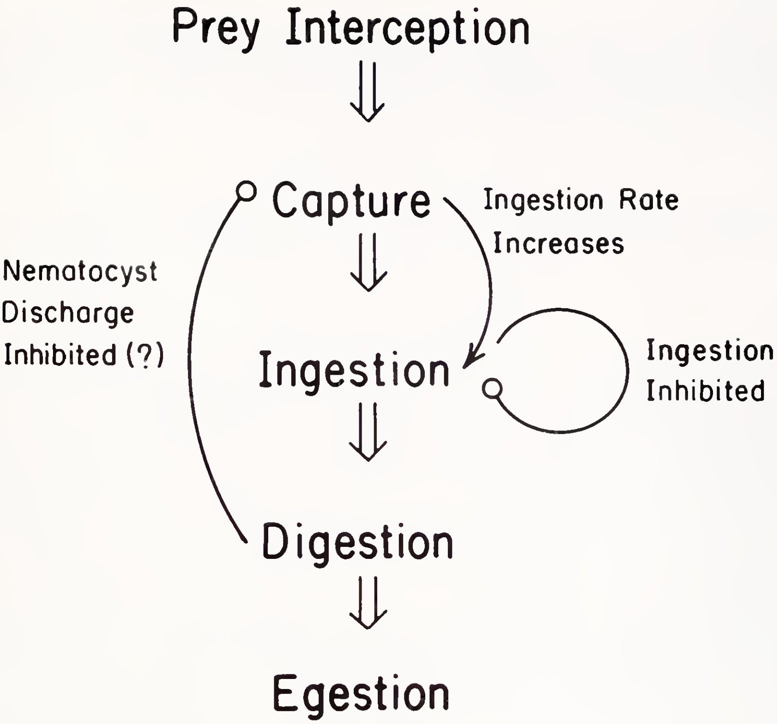

FIGURE 5. Generalized model of feeding showing effects of ingested prey on feeding. → indicates a stimulatory effect and ⊖ indicates an inhibitory effect.

As evident from Figure 4, at high prey densities *H. viridis* is capable of multiple captures, and an increase in the rate at which it ingests captured prey enables *H. viridis* to increase the number of prey consumed during a single capture–ingestion–digestion sequence.

The response of *H. viridis* to prey at different densities indicates that it is best suited to feeding on densely distributed prey. The enhanced ingestion rates reported here were observed at prey densities of 4,000 *Artemia* $\cdot l^{-1}$, while crustacean densities in lakes are usually in the range of 5–50 $\cdot l^{-1}$ (Wetzel, 1975). Denser concentrations, however, do occur and may be a regular feature of aquatic environments. In a study of a dense *Hydra* population in Toolik Lake, Alaska, Cuker and Mozley (1981) captured up to 14,000 *Bosmina* per day in 0.125 m^2 emergence traps. They report that the *Bosmina* were found in dense swarms and cite one example of *B. longirositis* swarms of up to 27,000 $\cdot l^{-1}$. Swarming has also been observed in *Heterocope septentrionalis* (Herbert, *et al.*, 1980) and is frequently observed in marine zooplankton which concentrate at the bottom during the day (Alldredge and King, 1977). As predicted, in the presence of plankton swarms *Hydra* are effective predators. Cuker and Mozley (1981) regularly found *Hydra* with 5–7 prey per gut and report one case of a *Hydra* with 23 carapaces in its gut. (The species Cuker and Mozley [1981] studies is much larger than *H. viridis*, which can hold only 5–7 *Artemia* in its gut.)

At low prey densities the simple gut and the feeding sequence exhibited by *Hydra* reduce their capability to consume prey. However, when prey are present in high density *Hydra* are capable of consuming large numbers of prey. Their ability to increase ingestion rates when many prey are captured further enhances their ability to feed and suggests that *Hydra* are adapted to feeding upon zooplankton found in dense swarms along the bottom.

ACKNOWLEDGMENTS

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TEMPORAL VARIATION IN THE REPRODUCTIVE CYCLE OF *MYTILUS EDULIS* L. (BIVALVIA, MYTILIDAE) FROM LOCALITIES ON THE EAST COAST OF THE UNITED STATES[1]

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ABSTRACT

The reproductive condition of seven latitudinally separated populations of the mussel *Mytilus edulis* on the east coast of the United States was determined using histological analysis and stereology. Differences in the timing of various phases of the gametogenic cycle among populations did not have any discernible latitudinal trend. Two populations at the same latitude on Long Island, N. Y. had the greatest temporal differences observed in gametogenic cycle, with summer reproduction maxima separated by a 3-month interval. There was no difference in the water temperature regime between these two habitats and thus the rate of gametogenic development was not a constant function of temperature. The observed differences in the gametogenic cycle were attributed to temporal and quantitative differences between habitats in the energy content of the mussel's food supply.

INTRODUCTION

Marine benthic bivalves have a cyclical pattern of reproduction which can be divided into three phases: gametogenesis and vitellogenesis; spawning and fertilization; larval development and growth. Each species has a variety of adaptations, both genetic and nongenetic, which coordinate these reproductive events with the environment so as to maximize reproductive success. It has been well documented (for review see Sastry, 1979) that the duration of each stage of the cycle may be variable among species and the gametogenic cycle may either be annual, semiannual, or continuous depending upon the species and environment. Although the reproductive cycles of numerous bivalve species have been described (reviewed by: Giese, 1959; Sastry, 1975, 1979; Seed, 1976; Andrews, 1979) there is still only partial understanding of the complex interactions between exogenous (*e.g.* food availability, temperature, salinity, *etc.*) and endogenous (nutrient reserves, hormonal cycle, genotype, *etc.*) variables that determine the initiation and duration of the various phases of the cycle, and thus ensure synchrony of gamete development within a population. This is of prime importance for dioecious species having external fertilization, which requires synchronized liberation of gametes.

Gametogenesis in *Mytilus edulis* is, in common with other boreo-temperate marine bivalves, a complex succession of events that eventually results in the for-

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Abbreviation: GVF, gamete volume fraction.

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mation of ripe gametes. Spawning is then followed by a period of reproductive recession and quiescence. Superimposed on the reproductive cycle is a nutrient storage cycle involving the accumulation of nutrients in the mantle which are mobilized for gametogenesis and for maintenance metabolism in the digestive gland (Gabbot, 1976).

In *M. edulis* which has a geographically broad distribution, in diverse habitats, the reproductive cycles of spatially separated populations differ (Seed, 1976). It is thus a suitable species in which to investigate the factors regulating the reproductive cycle by comparative studies of different populations.

This paper describes the reproductive cycles of two populations of *Mytilus edulis* on the north and south shores of Long Island, N. Y. We found such unexpectedly large differences in the gametogenic cycle of *M. edulis* between these two study sites that additional samples were collected from localities along the east coast of the United States in order to estimate the range of reproductive patterns found in geographically separated mussel populations. The documented differences in reproductive cycles will be related to certain environmental parameters in this report and to more detailed physiological cycles in subsequent publications.

Materials and Methods

Animal collection

The two main study areas were located on the north shore of Long Island, New York, at Stony Brook and on the south shore, in Shinnecock Bay (Fig. 1). The Stony Brook population was situated between Mean Low Water Springs (M.L.W.S.) and mid-tide on a sheltered beach, at the confluence of Stony Brook Creek, which drains a tidal marsh, and Long Island Sound. The Shinnecock population was an intertidal mussel bed located between M.L.W.S. and mid-tide level in a lagoon, formed between Long Island and the Atlantic barrier beach. Animals, larger than 2.5 cm in shell length, were collected from both populations at approximately monthly intervals, between June 1976 and September 1979. On each sampling occasion salinity and temperature were recorded with a Beckman salinometer, and 10 l of seawater was collected and analyzed for total energy content, using the dichromate wet oxidation method (Newell, 1982).

The remaining five sampling areas were on the east coast of the United States (Fig. 1). Detailed environmental data are not available for these localities, but each was selected as being representative of a typical and viable eulittoral population of *Mytilus edulis* in that region. The only exception was the population from Narragansett Bay, which was approximately 0.3 m below mean low tide. Samples were taken from these populations only at intervals between April and October 1978, as indicated in Figure 1.

Measurement of reproductive condition

A piece of germinal tissue ($\simeq 7 \times 4$ mm) was dissected from the same position in the gonad of each of 12 mussels. The tissue was fixed in Baker's formol calcium (+2.5% NaCl) for 24 h at 4°C prior to processing through an ascending alcohol series and embedding in paraffin wax. Two 7 μm sections, cut from different depths within the block, were stained with haematoxylin and counterstained with eosin. The mussels' reproductive condition was assessed by stereology (Bayne *et al.*, 1978) and expressed as the "gamete volume fraction" (GVF), which is the proportion of the mantle tissue that is composed of follicles containing developing or ripe gametes.

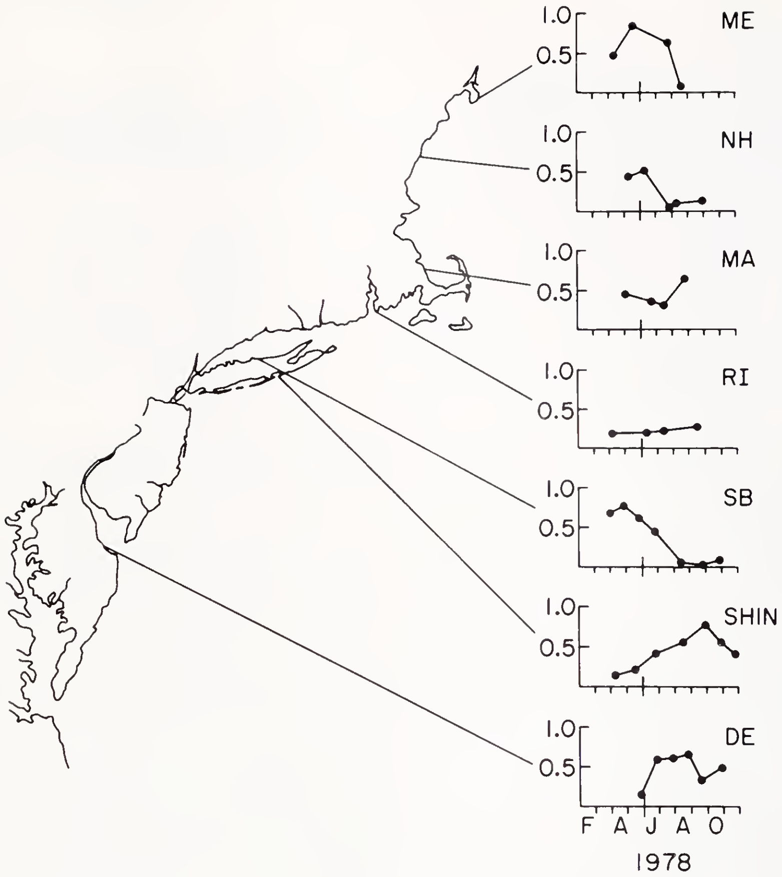

FIGURE 1. Mean reproductive condition for male and female *Mytilus edulis* from seven sites on the east coast of the United States. The reproductive condition, measured using stereology and expressed as Gamete Volume Fraction (G.V.F.) varies between 1.0 for a maximally ripe individual and 0.0 for an individual with no discernible gametes. Each point is a mean value calculated from 12 mussels (see text for further details). ME = Damariscotta River, Maine; NH = Newcastle, New Hampshire; MA = Cape Cod Canal, Massachusetts; RI = Narragansett Bay, Rhode Island; SB = Stony Brook, New York; SHIN = Shinnecock, New York; DE = Broadkill Inlet, Delaware.

Spaces within follicles which were observed when the mussels were maximally gravid were also counted in the GVF index. This was because the loss of gametes from these follicles probably only occurred either shortly before, or during the sampling process because the pressure from the rest of the gonad had not yet collapsed the intrafollicular space. The GVF can vary between zero, for a completely reproductively quiescent mussel, and one, for a mussel at peak reproductive condition. In this study, the mean of 10 estimates of the GVF of each animal (5 from each slide, cut from two different depths) was calculated and arcsine transformed (Sokal and Rohlf, 1969). Stereology is less subjective than the "index of bivalve gonad maturity" scheme proposed by Chipperfield (1953), where gonad squashes or stained sections are microscopically examined and assigned an arbitrary number (*c.f.*, Seed, 1976). Also, the Chipperfield technique does not fully recognize intermediate stages of development so the index results in nominal, rather than interval, measurements.

Results

Long Island populations

The energy content of the seston at Stony Brook, expressed as joules per liter was consistently greater during the summer (May to October) than at Shinnecock (Fig. 2). During the remainder of the year there were sharp fluctuations in the energy content of the seston at both localities.

The mean salinity at Stony Brook was 24.2‰ ± 2.0‰ (S.D.) and the temperature range was −0.5°C to 26.5°C (Fig. 3). Mean salinity (29.1‰ ± 1.3‰) was higher at Shinnecock, although the temperature range (Fig. 3) was very similar (0.8°C to 25.5°C) to that of Stony Brook. There is little tidal, and low annual, variation in salinity at these two localities (R.I.E. Newell, unpublished data), because neither area is associated with a river drainage system; most precipitation leaves Long Island by percolation through the sandy soil.

The reproductive condition (GVF) of male and female mussels from each population was analyzed separately to determine synchrony of their cycles. The mean GVF of each sex was compared using a Wilcoxon signed ranks test (Sokal and Rohlf, 1969) for all samples taken during a maturation phase (from the minima to maxima GVF) of the gametogenic cycles for each population; this tests the hypothesis that the same sexes mature at different rates within a population. In general, males had a higher GVF than did females during maturation; the difference was significant for the Shinnecock population ($P < 0.01$) but was not significant at Stony Brook ($P > 0.05$). Similar observations on the relative rates of reproductive maturation between sexes in the bivalves have been made for *M. edulis* (Seed, 1976) and *Cardium edule* (Newell and Bayne, 1980).

To facilitate comparisons of the average reproductive cycle of mussels from both populations, all male and female GVF valves were combined into a mean with 95% confidence limits for each sampling period (Fig. 4). Inspection of Figure 4 reveals that the reproductive cycle for *Mytilus edulis* at Stony Brook was constant between years in both the timing of the various phases of reproduction and the maximum reproductive condition attained. The post-spawning minimum GVF was in August/September, followed by a short period of reproductive quiescence from

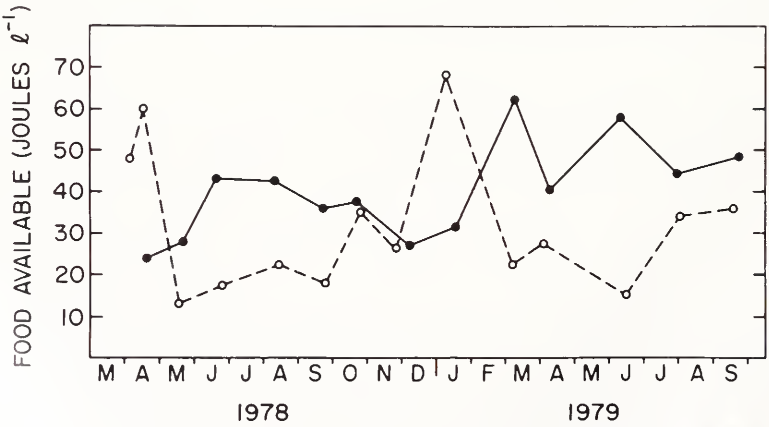

FIGURE 2. Seasonal changes in food availability (joules·l^{-1}) at Stony Brook (● —— ●) and Shinnecock (○ - - - ○). Each point is the mean of 2 replicates.

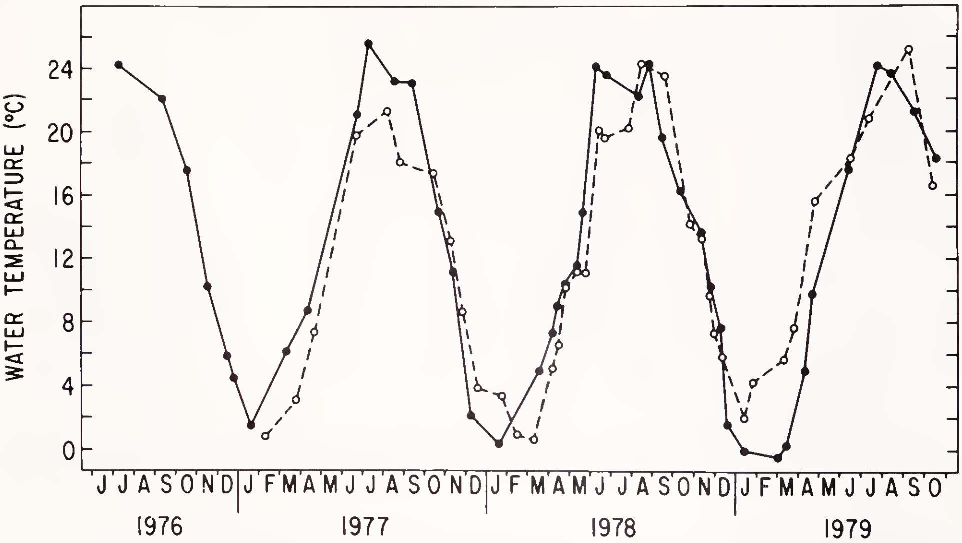

FIGURE 3. Seasonal variation in water temperature (°C) at Stony Brook (● —— ●) and Shinnecock (O - - - O). Each point represents a single measurement of water temperature.

September to October. Gametogenesis was initiated again in November and continued through the winter, with a peak GVF of approximately 0.8 in April/May.

In contrast, the Shinnecock population appears to have a less regular cycle, with large between-year variation in both the timing of various phases of the gametogenic cycle and the annual maximum reproductive condition attained (Fig. 4). The maximum GVF varied from about 0.45 GVF (1977) to 0.8 GVF (1978), generally lower than at Stony Brook. In each year, maximum GVF occurred between July and October, or about three months later than at Stony Brook. Shinnecock animals exhibited a minimum GVF between March and May of approximately 0.12 to 0.25 compared to about a 0.05 GVF in September and October for Stony Brook mussels. The gradual decline in GVF during the autumn and winter in the Shinnecock mussels was probably not associated with the liberation of gametes by spawning, but rather due to their reabsorbtion by phagocytosis. Evidence in support of this was an increased number of haemocytes in the interfollicular tissue. The post-spawning quiescent period was maintained until about May in the Shinnecock mussel, which is considerably longer than in the Stony Brook population.

A more subtle difference between the two populations of *Mytilus edulis* was the degree of synchrony of maximum gametogenic condition among individuals within each population. In general, the variance in GVF estimated from the 12 mussels from each sampling period was greater in the Shinnecock population (Wilcoxon two sample test, $P < 0.001$) indicating a greater degree of intrapopulation synchrony in gametogenesis for the Stony Brook population. These differences are apparent when the mean and variance of GVF are illustrated together (Figs. 5 and 6). Stony Brook mussels were closely synchronized (*i.e.* lowest variance) during the entire period of gametogenesis, but as spawning proceeded individuals became asynchronous, indicating some variation in the time taken to liberate all gametes completely. The Shinnecock individuals were generally most asynchronous (*i.e.* highest variance) just prior to, and during, the period of maximum reproductive

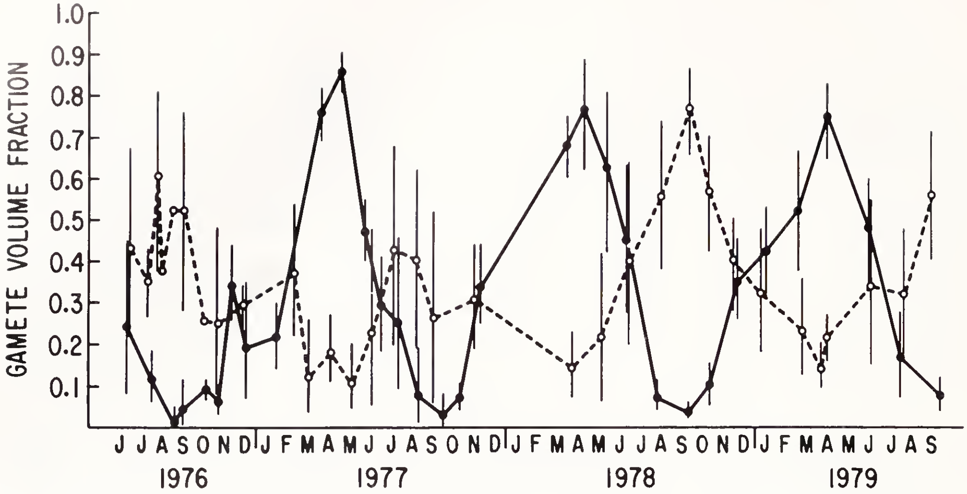

FIGURE 4. Mean reproductive condition (GVF) ± 95% confidence limits for male and female *Mytilus edulis* combined from Stony Brook (● —— ●) and Shinnecock (○ - - - ○).

condition. This indicates that the Shinnecock mussel population was spawning over a protracted period.

In order to evaluate the dependence of gametogenic state upon an interaction of temperature and time, the number of cumulative degree-days were determined for each sample period by calculating areas under the curves in Figure 3 using a Spatial Data Systems (108 pt) image analyzer. The number of degree-days elapsed at each sample date since the GVF minima (ending with GVF maxima) was estimated. Since cumulative degree-days were calculated starting with the annual minima in GVF, this analysis dictates a positive regression of GVF on elapsed degree-days; the comparison of interest is in the slopes of these regressions. If the gametogenic state of the two populations is largely dependent upon elapsed degree-

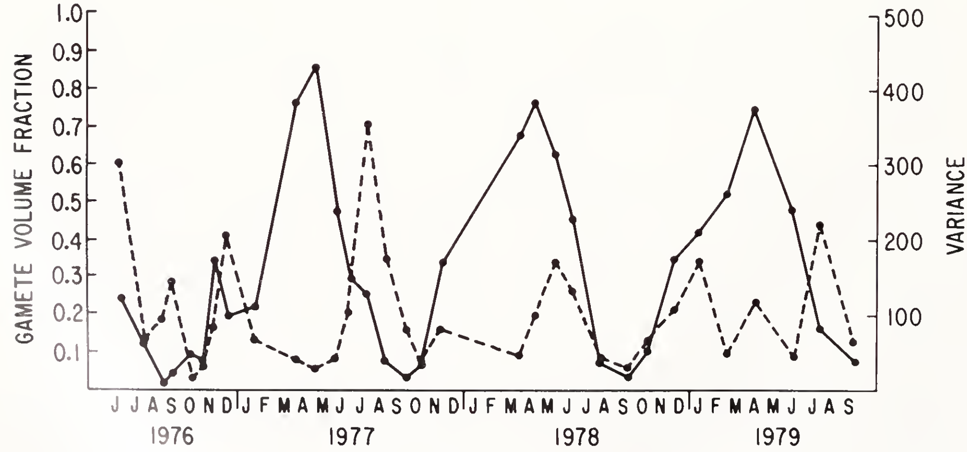

FIGURE 5. Mean reproductive condition (GVF; ● —— ●) and variance (● - - - ●) for male and female *Mytilus edulis* from Stony Brook.

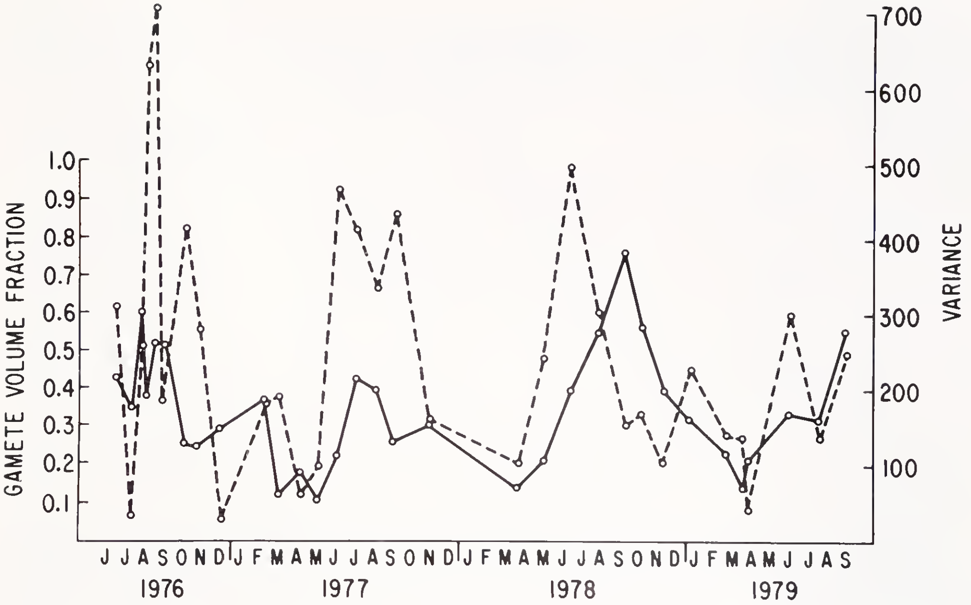

FIGURE 6. Mean reproductive condition (GVF; O——O) and variance (O – – – O) for male and female *Mytilus edulis* from Shinnecock.

days, the slopes will be similar. Analysis of covariance revealed heterogeneous slopes when all four regression lines were compared ($F_{\{3,9\}} = 10.25$; $P < 0.005$; Fig. 7). A simultaneous test procedure (Sokal and Rohlf, 1969) demonstrated that within population regressions between years had similar slopes while the slopes between populations were clearly different. This analysis suggests that differences in gametogenic cycles between the Stony Brook and Shinnecock populations were not directly related to any difference in schedule of degree-days (*i.e.* temperature).

N.E. coast populations

The mean reproductive condition of combined males and females from seven localities (including Stony Brook and Shinnecock) along the N.E. Atlantic Coast demonstrates large variation in the timing of maximum GVF (Fig. 1). This ranged from a peak in reproductive condition in May (Maine) to a peak in September (Shinnecock). The Rhode Island population had no obvious reproductive maxima during the six summer months. The differences between Stony Brook and Shinnecock, described earlier, were greater than between any other two sampled localities. There was no clear latitudinal trend in the timing of maximum GVF.

DISCUSSION

There have been many attempts to determine the key environmental factor(s) that synchronize the reproductive cycle among individuals within marine invertebrate populations to prevailing environmental conditions. Water temperature, which varies with season and latitude in a moderately uniform manner, frequently has been assigned a dominant synchronizing role. It has been found for certain species that the reproductive cycles of latitudinally separated populations were predictably different (Orton, 1920; Thorson, 1946). Thus a northern hemisphere boreal-tem-

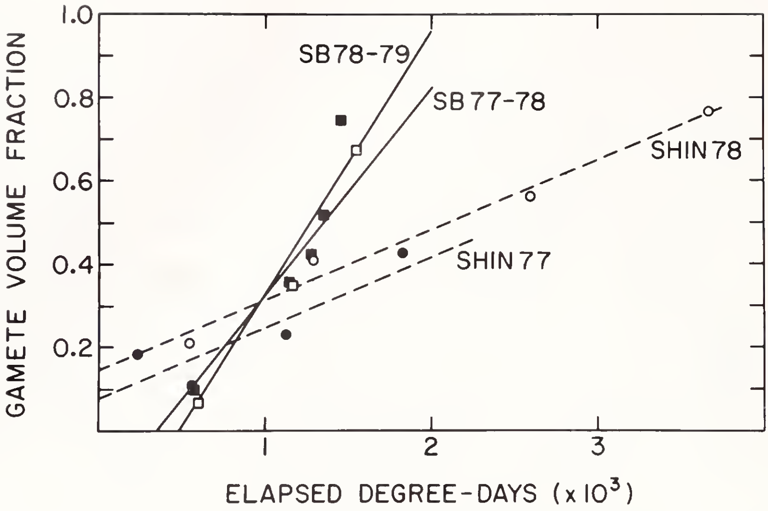

FIGURE 7. Least squares regression lines relating reproduction condition (GVF) to temperature, as measured by degree-days, at Stony Brook (SB; □, ■) and Shinnecock (SHIN: ○, ●), in the years indicated. The coefficient of determination (r^2) for the fitted lines are □ = 0.96; ■ = 0.86; ● = 0.89; ○ = 0.98.

perate species would spawn earlier and for a more extended period in the southern part of its range than in the northern part. From a review of the literature, Seed (1976) suggested that "mussels from the warmer more southerly waters of the northern hemisphere generally spawn earlier than those further north," which supports the general temperature/latitude zoogeographic principle outlined above.

The results of our study, however, do not corroborate such a generalization as there did not seem to be a clear trend in the timing of the maximum reproductive condition of *Mytilus edulis* over the range of temperate latitudes studied (Fig. 1). Indeed, the two mussel populations with the largest difference in reproductive cycle were from Long Island, New York, at Stony Brook and at Shinnecock, where the annual water temperature profiles (Fig. 3) were nearly identical. The absence of any influence of temperature, expressed as degree-days, on the reproductive cycle of the two mussel populations is in contrast to the findings of Bayne (1975) for mussels in the United Kingdom. He recorded in *M. edulis* a linear relationship between the rate of gametogenesis and degree-days, but not calendar days. However, even our results seem to indicate that gametogenesis is a function, albeit with a different exponent, of temperature. This relationship probably simply reflects the fact that the increase in water temperature at both localities was regular and temporally stable between years. Thus, degree-days are simply measuring time in the same fashion as calendar days.

Although a correlation between latitudinal differences in water temperature and the reproductive cycle has been documented for a number of species (Giese and Pearse, 1974), it is difficult to demonstrate a simple causal influence of temperature on reproduction. For example, food available to suspension feeders is dependent on environmental factors such as light availability leading to changes in plankton growth rates which covary with water temperature. The weight of evidence (reviewed by Giese, 1959; Sastry, 1975, 1979) does support the idea that a threshold temperature, or possibly a rate of change in temperature (Chipperfield, 1953; Bayne, 1975), is a very important factor influencing gametogenesis and acting as a trigger for spawning. However, gamete production is ultimately dependent on the nutrients available for gametogenesis, either in terms of a nutrient reserve or food recently ingested.

Mytilus edulis is normally dependent on a nutrient reserve, accumulated during the immediate post-spawning period (Gabbot, 1976) when food is abundant, to provide energy for gametogenesis and catabolism during the winter. Spawning then is usually timed such that both larvae and adults have access to abundant supplies of food; this maximizes the probability of successful recruitment and the rate of energy acquisition by adults for the following reproductive season (Bayne, 1976; Sastry, 1979). It is evident that environmental variation between habitats resulting in altered availability of food or the individual's capacity to assimilate nutrients will alter the nutrient storage cycle and the timing of gametogenic events.

There were distinct differences in both the amount and seasonal availability of energy to the Stony Brook and Shinnecock populations (Fig. 2). At Stony Brook food is most abundant in the late spring and summer, when mussel feeding rates are at a maximum, leading to the occurrence of maximal assimilation rates (R.I.E. Newell, unpublished data). Mussels at Stony Brook spawn in April/May just prior to, or during, the period of maximum food availability (Fig. 2). They can obtain sufficient nutrients from the seston during this post-spawning period to accumulate a food reserve as is considered typical for the species (Gabbott, 1976). In contrast, the Shinnecock population faces low levels of energy in the seston and unpredictable and sharp temporal peaks in energy availability during the same time of the year. The maximum seston energy levels were recorded during the winter when feeding rates are depressed (R.I.E. Newell, unpublished data). These factors lead to reduced total assimilation. Thus the high degree of synchrony in maximum gametogenic condition (and cyclicity) within the Stony Brook population reflects the timing and temporal variation in local food availability and of the long period during the winter in which gametogenesis may proceed. In the Shinnecock population, because maximum food availability occurs when the animals are not actively feeding, there is no period of maximum energy assimilation to give rise to a nutrient storage cycle. Instead, two possibilities exist: (1) individuals may initiate a "typical" gametogenic cycle once sufficient nutrient reserves are accumulated, but if environmental cues are lacking, individuals in the population are not synchronized, or (2) individuals develop gametes and hence spawn piecemeal as soon as sufficient reserves are gathered. Both of these conditions constitute "dribble spawning" with respect to the population.

Annual differences in maximum reproductive condition and fecundity have been previously noted in a *M. edulis* population by Thompson (1979) who attributed the differences to annual variations in the food supply in coastal inlets of Nova Scotia. He also found no differences in fecundity between 1974 and 1975 in the Stony Brook population, which supports our observation of a very constant maximum gamete volume fraction over the three year study period.

Griffiths (1977) found that in South African populations of *Choromytilus meridionalis*, which do not accumulate large nutrient reserves, the quantity of gametes released varied annually. Griffiths (1977) concluded that "food availability may be of greater importance in maturation of the gonad than temperature". This supports the argument propounded by Bayne (1976) that gametogenesis in some species is buffered from environmental change by a nutrient reserve. In other more "opportunistic" species, gametogenesis is more closely linked to the current food supply which allows them to capitalize on particularly favorable environmental conditions, *e.g.*, high phytoplankton production. However, the results of our study indicate that there may not be such a single reproductive "strategy" for a species but rather a variety of different patterns, depending on the particular environmental regime.

It is interesting to speculate on the adaptative significance of a peaked spring spawning period, compared to an extended one for planktotrophic mollusc species when similar numbers of gametes are liberated (Todd and Doyle, 1981). In the former case there is a highly probability of fertilization of the egg (Sastry, 1979). Also, larvae may be produced at the most opportune moment with regard to food availability (Sastry, 1975) and still have sufficient time both to outgrow predation rapidly (Seed and Brown, 1978) and accumulate a nutrient reserve before the onset of winter. In the latter case there is continuous dribble spawning, and hence prolonged recruitment, which means that in the event of a catastrophy in an unpredictable environment, which could kill or prevent settlement of the vulnerable larvae, only a portion of the potential recruits would be lost. Our data suggest that for sessile invertebrates neither pattern may necessarily be adaptative but may simply be a manifestation of variation in exogenous factors.

The temporal differences in the reproductive cycles of adjacent *M. edulis* populations recorded in this study have also been noted for the species by Moore and Reisch (1969) within Alamitos Bay, California. However, the environmental cause of these differences could not be determined. Also, Wilson and Hodgkin (1967) concluded that although temperature probably controlled the overall reproductive cycle (*i.e.* duration and season of gametogenic activity) in five species of mytilids on the western Australian coast, some unknown factor regulated the finer detail of the cycle (*i.e.* the intensity and duration of spawning and exactly when it occurred).

Bayne (1975) in a comprehensive review of reproduction in *M. edulis* concluded that both "temperature and the food level serve to synchronize different stages in the gametogenic cycle." Blake and Sastry (1979) found that temperature and nutrient availability interact with neurosecretory cycles of *Argopecten irradians* in a complex manner to control the initiation of gametogenesis. The nature of these interactions changed as the reproductive cycle proceeded. Similarly Sastry (1970) found that a population of *Argopecten irradians* from North Carolina reached its reproductive peak 3 months after a population from Massachusetts. This conflicts with the simple latitude/temperature zoogeographic principle discussed previously. Sastry (1970) suggested that the differences might be "an adaptative response to geographical differences in the period of abundant food production and temperatures."

Thus, superimposed on the overall affect of latitude (and hence by implication, water temperature) on the reproductive cycle of bivalves must be variations due to habitat-specific differences in the time and duration of maximum food availability. The animals' physiological adaptations must also be considered as they can potentially be adjusted to maximize the animals' fecundity and hence competitive ability in a particular environment.

Geographic variation in reproductive cycle, whatever the cause, has also been attributed to the existence of "physiological races" (Loosanoff and Nomejko, 1951), implying genetic differences among populations. Populations of *M. edulis* along the Atlantic coast of North America are known to be genetically differentiated (Koehn *et al.*., 1976; Koehn *et al.*, 1980), and the relationship between genetic differences and variation in the physiology of reproduction will be considered in a subsequent paper.

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A SURVEY OF THE RESPONSES OF BIVALVE HEARTS TO THE MOLLUSCAN NEUROPEPTIDE FMRFAMIDE AND TO 5-HYDROXYTRYPTAMINE

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Abstract

Ventricles from 50 species of bivalved molluscs were surveyed for their mechanical responses to the molluscan neuropeptide FMRFamide (Phe-Met-Arg-Phe-NH_2) and to 5-hydroxytryptamine (5HT). Both were predominantly cardioexcitatory, but neither was exclusively so. FMRFamide was inhibitory or weakly excitatory more often than 5HT, and such effects were most common in the subclasses Paleoheterodonta and Heterodonta. In contrast, 5HT was only rarely inhibitory or even weakly excitatory, and such effects were most common in the subclass Pteriomorphia. The responses to FMRFamide or 5HT were strikingly uniform in some bivalve families, but characteristically diverse in others. Thus, FMRFamide is neither a general cardioexcitor nor a general serotonomimetic agent.

Introduction

The molluscan neuropeptide FMRFamide (Phe-Met-Arg-Phe-NH_2), isolated from the clam *Macrocallista nimbosa*, increases the force and frequency of beat of isolated *Macrocallista* or *Mercenaria mercenaria* ventricles. These actions are identical to those of 5-hydroxytryptamine (5HT; serotonin) (Price and Greenberg, 1977). Both FMRFamide and 5HT stimulate adenylate cyclase activity and elevate cyclic adenosine monophosphate (cAMP) levels in these hearts (Higgins, 1977; Higgins *et al.*, 1978), even though the two agonists act at pharmacologically distinguishable receptor sites (Price and Greenberg, 1977). These observations suggested that FMRFamide might function as a long distance, long duration serotonomimetic agent (Price and Greenberg, 1977).

To test this hypothesis, we began to survey the effects of FMRFamide and 5HT on the mechanical activity of hearts from many species of bivalved molluscs. It soon became clear that FMRFamide is neither an unswerving mimic of 5HT nor an inevitable cardioexcitor. These general conclusions and representative data have been presented to the American Society of Zoologists (Painter *et al.*, 1979) and reviewed (Greenberg and Price, 1979, 1980; Greenberg *et al.*, 1982), but the survey itself, now completed, has never been published. These primary data are presented here.

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Abbreviations: FMRFamide, Phe-Met-Arg-Phe-NH_2 (from the one letter abbreviations for amino acids approved by the IUPAC-IUB Commission on Biochemical Nomenclature, and after Price and Greenberg, 1977); 5HT, 5-hydroxytryptamine; DRP, dose-response profile; SRI, species response index.

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MATERIALS AND METHODS

Animals

Most freshwater bivalves were obtained from the Ochlockonee River and Lake Talquin in Leon County, Florida; but *Ligumia recta* and *Lampsilis ovata ventricosa* were collected from the Wisconsin River in Richland County, Wisconsin, and *Ligumia subrostrata* from a pond in Livingston Parish, Louisiana. Gulf coast marine and brackish water animals were taken from the estuaries, marshes, and sand bars of north Florida. Bivalves of the northeastern Atlantic coast were purchased from Northeast Marine Specimens Co. of Bourne, Massachusetts; Pacific coast animals from Pacific Bio-Marine Co. of Venice, California; and *Lima scabra* from Gulf Specimens Co. of Panacea, Florida. Most Panamanian species were purchased at a fish market in Panama City, Panama; *Ostrea palmula* were collected from Miraflores Third Locks Lake.

All animals were maintained in aerated aquaria for several days before use. The marine species were kept in seawater, the brackish water bivalves in diluted seawater (375 mOsm for *Ostrea palmula*, 300 mOsm for both *Rangia cuneata* and *Polymesoda caroliniana*), and the freshwater animals in river water. The osmotic concentrations of the media were monitored with a freezing point depression osmometer (Precision Instruments, Osmette) and were replaced as needed. The Atlantic and Pacific coast animals were maintained and tested at 16°C, but all others at 21°C.

Procedure

The ventricles were prepared according to the procedures of Welsh and Taub (1948) and Greenberg (1965). One end of each heart was secured to a hook at the bottom of an organ bath, and the other end was attached via a spring to a force-displacement transducer (Grass Model FT-03). Mechanical activity was recorded on a Grass Model 79C polygraph.

The hearts of marine species were superfused with natural seawater, and the others with an appropriately diluted seawater: 50 mOsm for the freshwater species (Deaton and Greenberg, 1980), 300 mOsm for *R. cuneata* and *P. caroliniana*, and 375 mOsm for *O. palmula.* Aeration and mixing were provided by a magnetic stirring bar at the bottom of the bath.

Each heart was treated with a sequence of increasing doses of FMRFamide and 5HT. The drugs were added directly to the bathing medium, and the medium was changed between successive doses. The full range of FMRFamide concentrations was usually tested before 5HT; but we reversed the order of application in a number of preliminary experiments and found no effect on the responses to either agent. All doses are expressed as final molar concentrations in the bathing medium.

Chemicals

The drugs used in this study include: FMRFamide (Peninsula Labs), and 5-hydroxytryptamine creatinine sulfate (5HT) (Sigma).

RESULTS

This survey includes over 9000 responses which vary qualitatively with species, drug, and dose tested. The major problem has been to characterize and organize these disparate responses so that pharmacological and taxonomic comparisons could be made. We have approached the problem as follows.

Individual responses

To reduce the qualitative variation between individual responses, drug effects have been resolved into "excitatory" and "inhibitory" components, as detailed below.

Increases in frequency or diastolic tone were classified as excitations, while decreases were inhibitions. Frequency and diastolic tone usually changed in the same direction, although tone was affected mainly at higher doses.

Changes in amplitude were more difficult to assess since contractile force is often inversely related to frequency (molluscs: Greenberg, 1963; mammals: Blinks and Koch-Weser, 1961). Therefore, changes in amplitude were used to classify responses only when neither frequency nor diastolic tone were affected. In such cases, positive inotropy was excitation and negative inotropy inhibition.

An induced arrhythmia, although rare, was classified as an inhibition. An improvement in rhythmicity was more common, especially at threshold doses, and was designated an excitation.

Each response was thus classified as an excitation, an inhibition, or a mixed (*i.e.,* containing both excitatory and inhibitory components) response. Figure 1 clearly shows that FMRFamide and 5HT produced qualitatively similar excitatory, inhibitory, and mixed responses, though not necessarily in the same species or at the same concentration.

Dose-response profiles (DRPs)

A DRP is the set of responses of any preparation to a specified sequence of doses of agonist (1×10^{-10} to 1×10^{-6} *M*). Since most hearts were not exposed to doses exceeding 1×10^{-6} *M*, the effects of those high doses are not included in the profiles.

Some DRPs consisted entirely of qualitatively similar responses, either excitatory or inhibitory. Such profiles can be represented by a conventional dose-response curve and characterized by an ED_{50}. The individual responses of many DRPs changed qualitatively with dose, however, and these profiles are not amenable to the usual graphical representations. We had to develop new techniques to deal with these kinds of profiles.

Examples of FMRFamide and 5HT DRPs are illustrated in Figures 2 and 3, respectively. Each DRP was categorized as excitatory, inhibitory, or complex (*i.e.,* having both excitatory and inhibitory components, and changing qualitatively with dose) based on the sequence of effects observed.

Species responses

The species response is the set of DRPs to FMRFamide or 5HT obtained from a single species. If the species response is uniform, the same sequence of effects was observed in each preparation; *i.e.,* the DRPs are similar. If the species response is diverse, the DRPs differed qualitatively among preparations. In either case, a preponderant effect (excitation or inhibition) is usually identifiable; when it is not, the species response is complex.

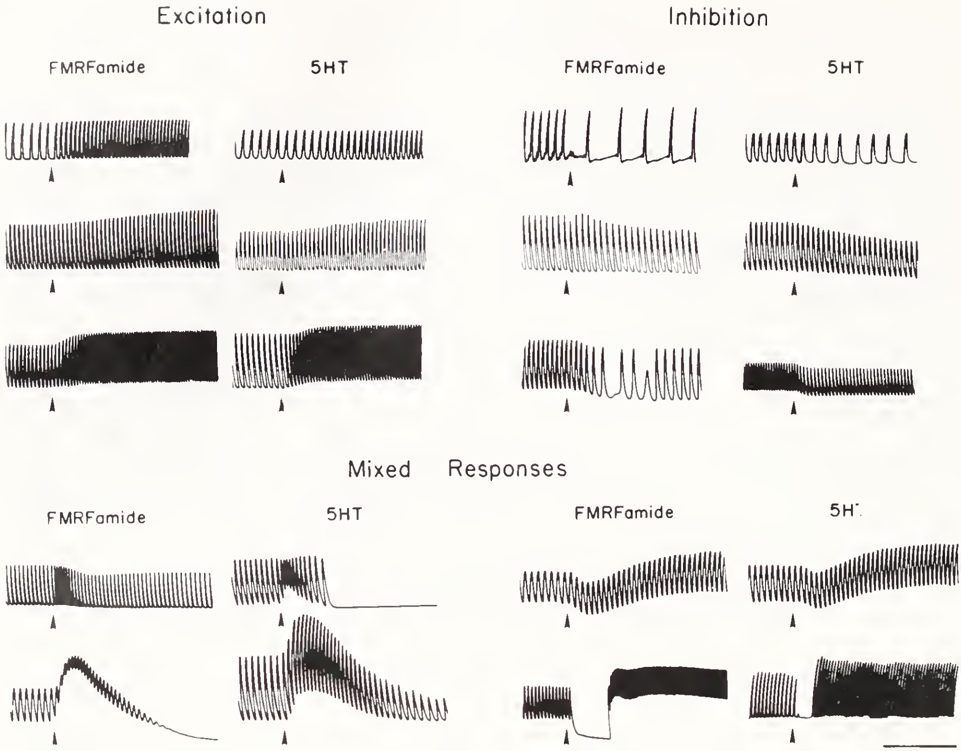


FIGURE 1. Examples of the responses of bivalve hearts to FMRFamide and 5HT. The species are identified from top to bottom and left to right. FMRFamide excitation: *Geukensia demissa granosissima*, 1×10^{-7} *M*; *Dinocardium robustum*, 3×10^{-7} *M*; *Modiolus squamosus*, 1×10^{-7} *M*. 5HT excitation: *Mytella guyanensis*, 3×10^{-7} *M*; *Lampsilis ovata ventricosa*, 1×10^{-6} *M*; *Mytilus edulis*, 1×10^{-6} *M*. FMRFamide inhibition: *Elliptio icterina*, 1×10^{-7} *M*; *Lampsilis ovata ventricosa*, 1×10^{-8} *M*; *Cyrtopleura costata*, 1×10^{-7} *M*. 5HT inhibition: *Geukensia demissa granosissima*, 1×10^{-8} *M*; *Lampsilis ovata ventricosa*, 1×10^{-5} *M*; *Rangia cuneata*, 1×10^{-6} *M*. FMRFamide mixed responses: *Rangia cuneata*, 1×10^{-7} *M*; *Pseudochama exogyra*, 1×10^{-6} *M*; *Corbicula manilensis*, 3×10^{-10} *M*; *Noetia ponderosa*, 1×10^{-5} *M*; 5HT mixed responses: *Villosa lienosa*, 1×10^{-5} *M*; *Lampsilis claibornensis*, 1×10^{-6} *M*; *Corbicula manilensis*, 3×10^{-9} *M*; *Anadara tuberculosa*, 3×10^{-7} *M*. Drugs were added to the bath at the arrows. Time: 1 min.

Tables and plots

Tabulating the effects. The individual responses making up each DRP were examined for excitatory and inhibitory component effects, and the threshold for each effect was determined. Thresholds, rather than ED_{50}s, were recorded because the effects changed qualitatively with dose in many preparations. The data were compiled by species and are presented in Table I. The percentage of responding preparations affected in each of the two modes and the number of preparations failing to respond in either mode are indicated for each species. Unresponsive hearts were challenged with very high doses of agonist ($1–3 \times 10^{-5}$ *M*) and these responses (or lack of them) are also recorded in Table I.

Table I thus provides the initial characterization of the species responses to FMRFamide and 5HT. Subsequent derivations are intended to illustrate patterns among the species responses which are not immediately apparent from this characterization.

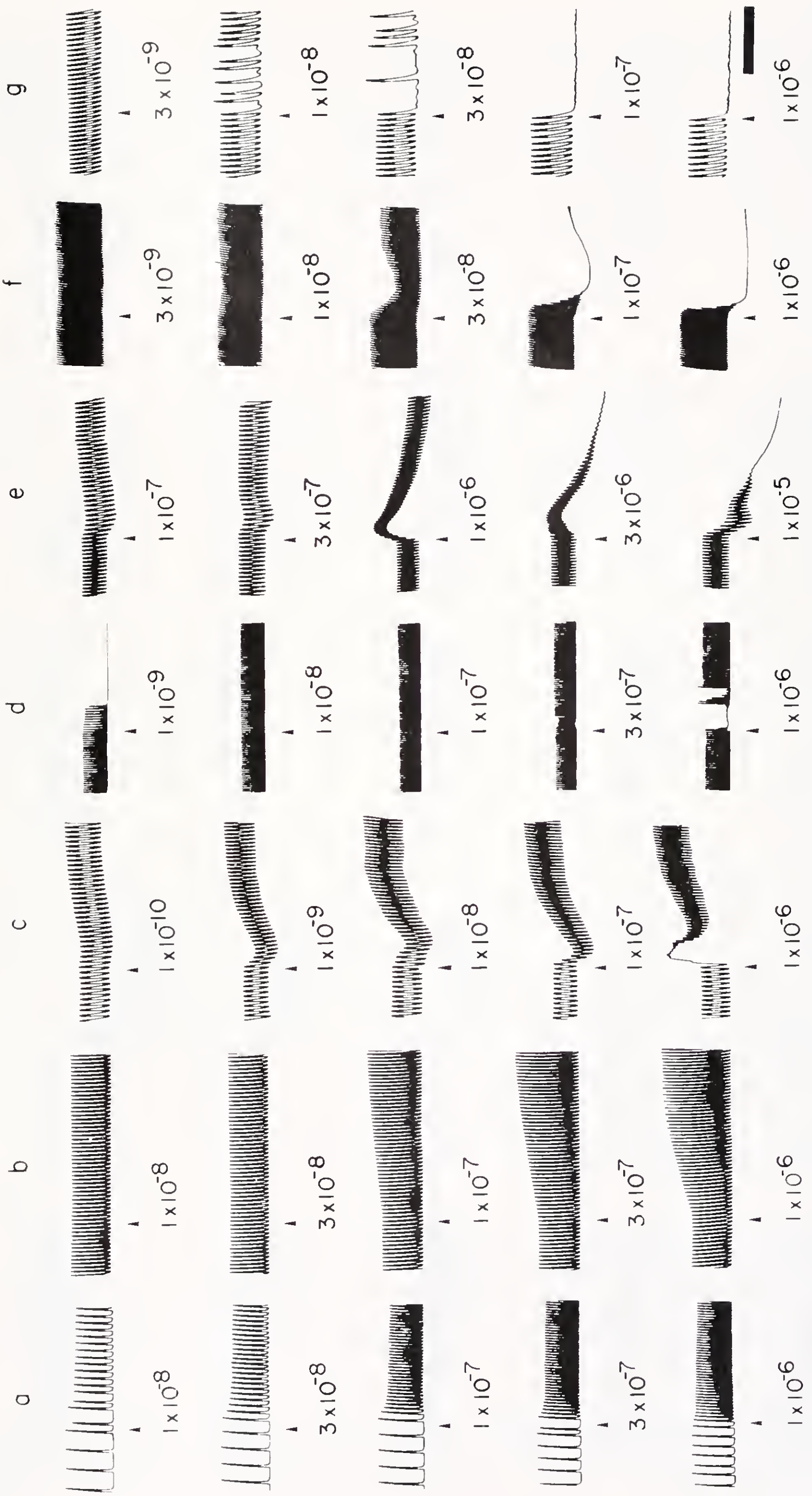
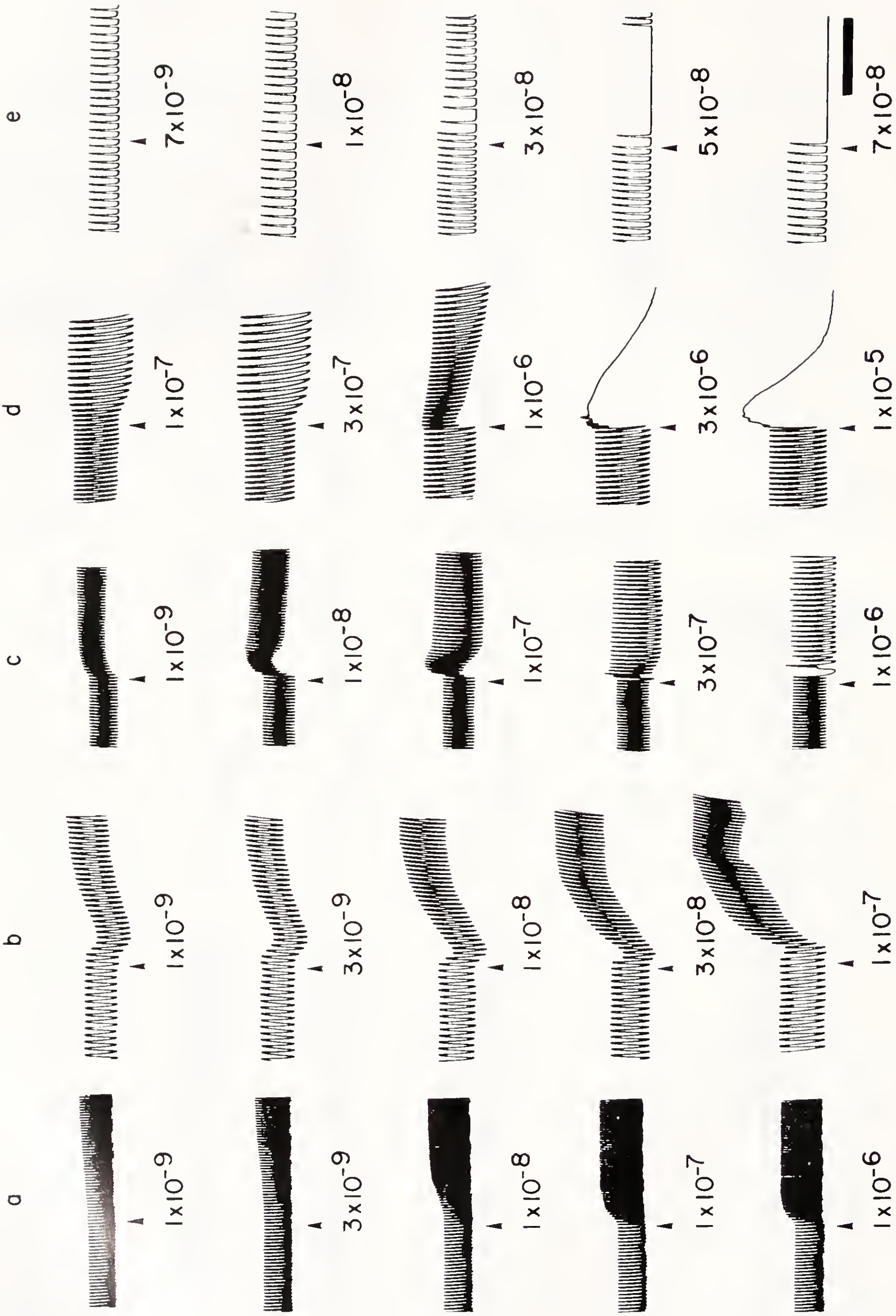


FIGURE 2. Examples of FMRFamide dose-response profiles (DRPs); each profile is from a different species. Drugs were added to the bath at the arrows; all doses are final bath concentrations. a) *Geukensia demissa granosissima.* b) *Dinocardium robustum.* c) *Corbicula manilensis.* d) *Atrina rigida.* e) *Pseudochama exogyra.* f) *Trachycardium egmontianum.* g) *Lampsilis claibornensis.* Time: 1 min.


a
1x10-9
3x10-9
1x10-8
1x10-7
1x10-6
b
1x10-9
3x10-9
1x10-8
3x10-8
1x10-7
c
1x10-9
1x10-8
1x10-7
3x10-7
1x10-6
d
1x10-7
3x10-7
1x10-6
3x10-6
1x10-5
e
7x10-9
1x10-8
3x10-8
5x10-8
7x10-8

Species response index (SRI). The SRI is a shorthand description of the species response and is derived from the distribution of DRPs among the three categories of effects outlined above (*i.e.,* excitatory, inhibitory and complex). This distribution (and therefore the SRI) is expressed in two terms. The first is the number of DRP categories observed (*i.e.,* 1–3), and this is a measure of intraspecific diversity. The second term describes the predominant effect: "e" if there were more excitatory than inhibitory DRPs, "i" if there were more inhibitory DRPs, and "c" if there were equal numbers of excitatory and inhibitory DRPs (Table II). The taxonomic distribution of SRIs by subclass is summarized in Table III.

Mean response plot. For each species and each agonist, we calculated the percentage of doses within the range of 1×10^{-10} to 1×10^{-6} *M* that produced responses with excitatory or inhibitory components. The size of the effect was not considered in these calculations. We then graphed the percentage of inhibitory effects as a function of the percentage of excitatory effects and thereby produced mean response plots (Figs. 4 and 5). Each species response is reduced to a single point in these plots. Species responses lying on the X-axes were uniformly excitatory, and those on the Y-axes uniformly inhibitory. The further the point is from the origin, the larger the percentage of effective doses and the more sensitive the species was to the agonist.

Species response curves. For each species and each agonist, we also calculated the percentages of preparations excited and inhibited by each concentration tested, from 10^{-10} to 10^{-6} *M*. The percentages were plotted against the log of the concentration, generating species response curves (Fig. 6 shows seven of them). Each species response is represented by two curves, one for excitation and one for inhibition. Of course, if the species response is uniformly excitatory (for example), then the inhibitory line will lie at Y = O.

Comparison of analytical approaches

The three analytical approaches described above provide complementary information about the species responses. For example, the SRI identifies the effect most often observed in all of the preparations of a species, and it also characterizes the variation among the DRPs. But its reliability depends strongly upon sample size. That is, we often found that the diversity of the species response would increase as we tested more preparations. The mean response plots, in contrast, display the relative contributions of the excitatory and inhibitory components to the species response, and are convenient for comparative purposes. Nevertheless, they are limited because the points are averages and concentration-independent. Finally, the species response curves display the dose-distributions of the excitatory and inhibitory effects, but they are less convenient than the mean response plots for comparing species responses.

The complementarity of the approaches becomes evident when we use all three techniques to analyze the responses of seven species with complex DRPs to both FMRFamide and 5HT (** in Table II; Figs. 6 and 7). The three methods all show that three species (*Anadara ovalis, Corbicula manilensis,* and *Macrocallista nimbosa*) have qualitatively similar responses to the two agonists, and that three others

FIGURE 3. Examples of 5HT dose-response profiles (DRPs); each profile is from a different species. Drugs were added to the bath at the arrows; all doses are final concentrations in the bath. a) *Modiolus squamosus.* b) *Corbicula manilensis.* c) *Rangia cuneata.* d) *Elliptio icterina.* e) *Geukensia demissa granosissima.* Time: a, c, d, e, 2.5 min; b, 1 min.

TABLE I

Summary of the excitatory (Exc) and inhibitory (Inh) responses of bivalve hearts to FMRFamide and 5HT.

SUBCLASS* Family Species (Number tested)	FMRFamide Response (%)	 Threshold range (*M*)	 U†	5HT Response (%)	 Threshold range (*M*)	 U†
PTERIOMORPHIA						
Arcidae						
Anadara lienosa floridana (2)	Exc (100)	3×10^{-9}–3×10^{-8}	0	Exc (100)	3×10^{-8}	0
Anadara tuberculosa (9)	Exc (100)	1×10^{-8}–3×10^{-7}	0	Exc (100) Inh (67)	3×10^{-9}–1×10^{-7} 3×10^{-9}–1×10^{-7}	0
Anadara ovalis (7)	Exc (100) Inh (100)	3×10^{-10}–1×10^{-7} 1×10^{-10}–1×10^{-8}	0	Exc (100) Inh (100)	1×10^{-9}–1×10^{-8} 1×10^{-10}–1×10^{-7}	0
Noetiidae						
Noetia ponderosa (7)	Exc (86) Inh (100)	1×10^{-9}–1×10^{-7} 3×10^{-9}–3×10^{-7}	0	Exc (100)	3×10^{-9}–1×10^{-7}	0
Mytilidae						
Mytilus edulis (9)	Exc (100)	3×10^{-8}–5×10^{-7}	0	Exc (100)	3×10^{-9}–3×10^{-8}	0
Mytella guyanensis (6)	Exc (100) Inh (33)	1×10^{-8}–1×10^{-7} 1×10^{-7}–3×10^{-7}	0	Exc (100)	1×10^{-9}–1×10^{-8}	0
Brachidontes recurvus (4)	Exc (100)	3×10^{-9}–3×10^{-8}	0	Exc (100) Inh (50)	1×10^{-9}–1×10^{-8} 1×10^{-7}–1×10^{-6}	0
Modiolus squamosus (7)	Exc (100)	1×10^{-9}–1×10^{-8}	0	Exc (100)	3×10^{-10}–3×10^{-9}	0
Geukensia demissa demissa (23)	Exc (100)	1×10^{-8}–3×10^{-7}	0	Exc (56) Inh (100)	3×10^{-9}–1×10^{-7} 3×10^{-9}–1×10^{-6}	0
Geukensia demissa granosissima (8)	Exc (100)	1×10^{-8}–5×10^{-8}	0	Exc (25) Inh (100)	1×10^{-8}–3×10^{-8} 3×10^{-9}–1×10^{-7}	0
Pinnidae						
Atrina rigida (10)	Exc (100) Inh (40)	3×10^{-9}–3×10^{-7} 1×10^{-9}–1×10^{-8}	0	Exc (100)	1×10^{-8}–1×10^{-6}	0
Pectinidae						
Argopecten irradians (8)	Exc (100)	3×10^{-9}–1×10^{-7}	1	Exc (100)	3×10^{-8}–1×10^{-7}	0

Limiidae						
Lima scabra (7)	Exc (100)	3×10^{-8}–3×10^{-6}	1	Exc (100)	1×10^{-8}–3×10^{-7}	0
Ostreidae						
Crassostrea virginica (31)	Exc (62) Inh (90)	3×10^{-7}–3×10^{-5} 1×10^{-6}–3×10^{-5}	10	Exc (100)	3×10^{-9}–1×10^{-6}	0
Ostrea palmula (7)	Inh (100)	3×10^{-6}–1×10^{-5}	2	Exc (100)	3×10^{-9}–3×10^{-7}	0
PALEOHETERODONTA						
Unionidae						
Lampsilis claibornensis (21)	Inh (100)	3×10^{-10}–3×10^{-8}	0	Exc (100) Inh (85)	3×10^{-10}–1×10^{-7} 3×10^{-8}–1×10^{-6}	0
Lampsilis ovata ventricosa (8)	Exc (62) Inh (88)	1×10^{-8}–1×10^{-6} 1×10^{-9}–3×10^{-7}	0	Exc (100)	1×10^{-9}–1×10^{-7}	0
Lampsilis teres (8)	Exc (100)	3×10^{-9}–3×10^{-7}	0	Exc (100)	1×10^{-9}–1×10^{-7}	0
Ligumia recta (8)	Exc (100)	3×10^{-9}–1×10^{-7}	0	Exc (100) Inh (50)	3×10^{-10}–3×10^{-8} 1×10^{-6}–1×10^{-5}	0
Ligumia subrostrata (9)	Exc (56) Inh (89)	1×10^{-7}–1×10^{-6} 1×10^{-7}–3×10^{-6}	0	Exc (100)	3×10^{-10}–3×10^{-8}	0
Villosa villosa (12)	Exc (100)	1×10^{-9}–1×10^{-8}	0	Exc (100) Inh (83)	3×10^{-10}–3×10^{-9} 3×10^{-8}–1×10^{-6}	0
Villosa lienosa (6)	Exc (100)	3×10^{-10}–1×10^{-8}	0	Exc (100) Inh (83)	3×10^{-10}–1×10^{-9} 3×10^{-8}–3×10^{-7}	0
Elliptio icterina (5)	Exc (100) Inh (100)	1×10^{-8}–1×10^{-6} 1×10^{-9}–3×10^{-8}	0	Exc (100) Inh (60)	1×10^{-9}–3×10^{-7} 3×10^{-8}–3×10^{-7}	0
Anodonta peggyae (4)	Exc (100)	3×10^{-8}–3×10^{-7}	0	Exc (100)	1×10^{-8}–3×10^{-8}	0
Anodonta cataracta (3)	Exc (100)	1×10^{-9}–3×10^{-9}	0	Exc (100)	1×10^{-9}–1×10^{-8}	0
Quincuncina infucata (5)	Exc (100) Inh (80)	1×10^{-9}–3×10^{-8} 3×10^{-7}–1×10^{-6}	0	Exc (100)	1×10^{-9}–3×10^{-8}	0
HETERODONTA						
Chamidae						
Chama pellucida (9)	Exc (100) Inh (33)	1×10^{-8}–3×10^{-7} 1×10^{-7}–1×10^{-6}	0	Exc (100)	1×10^{-9}–3×10^{-8}	0
Pseudochama exogyra (3)	Exc (33) Inh (100)	3×10^{-7} 3×10^{-9}–3×10^{-8}	0	Exc (100)	1×10^{-8}–1×10^{-7}	0

TABLE I (*Continued*)

SUBCLASS*	FMRFamide			5HT		
Family Species (Number tested)	Response (%)	Threshold range (M)	U†	Response (%)	Threshold range (M)	U†
Cardiidae						
Dinocardium robustum (9)	Exc (100)	1×10^{-8}–1×10^{-7}	0	Exc (100)	1×10^{-8}–3×10^{-7}	0
Trachycardium egmontianum (10)	Exc (30)	1×10^{-9}–1×10^{-8}	0	Exc (100)	1×10^{-9}–1×10^{-6}	0
	Inh (100)	1×10^{-9}–3×10^{-7}		Inh (70)	3×10^{-8}–1×10^{-4}	
Mactridae						
Rangia cuneata (21)	Exc (100)	1×10^{-9}–3×10^{-8}	0	Exc (100)	1×10^{-10}–1×10^{-8}	0
	Inh (90)	1×10^{-10}–1×10^{-7}		Inh (100)	3×10^{-9}–3×10^{-5}	
Spisula solidissima (10)	Exc (100)	3×10^{-9}–1×10^{-7}	0	Exc (100)	3×10^{-10}–3×10^{-9}	0
Tresus nuttalli (11)	Exc (100)	3×10^{-7}–3×10^{-6}	0	Exc (100)	1×10^{-9}–3×10^{-8}	0
	Inh (56)	1×10^{-8}–1×10^{-7}				
Solenidae						
Ensis directus (5)	Exc (100)	1×10^{-9}–1×10^{-7}	0	Exc (100)	3×10^{-9}–3×10^{-8}	0
	Inh (80)	1×10^{-9}–1×10^{-8}				
Semelidae						
Semele decisa (3)	Exc (100)	1×10^{-7}–1×10^{-6}	0	Exc (100)	3×10^{-8}	0
Semele rupicola (4)	Exc (75)	1×10^{-7}–3×10^{-6}	0	Exc (100)	1×10^{-8}–1×10^{-7}	0
	Inh (75)	1×10^{-7}–3×10^{-7}				
Solecurtidae						
Tagelus plebeius (7)	Exc (100)	3×10^{-9}–1×10^{-7}	0	Exc (100)	1×10^{-8}–3×10^{-8}	0

Corbiculidae						
Polymesoda caroliniana (4)	Exc (100)	3×10^{-10}–3×10^{-9}	0	Exc (100)	1×10^{-9}	0
Corbicula manilensis (12)	Exc (100)	1×10^{-10}–3×10^{-9}	0	Exc (100)	3×10^{-10}–1×10^{-9}	0
	Inh (67)	1×10^{-10}–3×10^{-9}		Inh (67)	3×10^{-10}–1×10^{-9}	
Veneridae						
Mercenaria mercenaria (8)	Exc (100)	3×10^{-9}–1×10^{-7}	0	Exc (100)	1×10^{-9}–3×10^{-8}	0
Mercenaria campechiensis (11)	Exc (100)	3×10^{-10}–3×10^{-9}	0	Exc (100)	3×10^{-10}–1×10^{-8}	0
Chione cancellata (10)	Exc (100)	3×10^{-9}–5×10^{-8}	0	Exc (100)	3×10^{-9}–1×10^{-7}	0
Protothaca asperrima (7)	Exc (86)	1×10^{-9}–3×10^{-9}	0	Exc (100)	3×10^{-9}–1×10^{-7}	0
	Inh (100)	1×10^{-9}–3×10^{-8}				
Tivela stultorum (7)	Exc (100)	3×10^{-8}–3×10^{-7}	0	Exc (100)	3×10^{-9}–3×10^{-8}	0
				Inh (28)	3×10^{-7}–1×10^{-6}	
Macrocallista nimbosa (26)	Exc (100)	1×10^{-9}–3×10^{-8}	0	Exc (100)	1×10^{-9}–1×10^{-7}	0
	Inh (8)	1×10^{-7}–1×10^{-6}		Inh (29)	1×10^{-7}–1×10^{-5}	
Saxidomus nuttalli (8)	Exc (100)	1×10^{-8}–3×10^{-8}	0	Exc (100)	3×10^{-9}–3×10^{-8}	0
Dosinia discus (26)	Exc (88)	1×10^{-7}–1×10^{-5}	0	Exc (100)	3×10^{-9}–3×10^{-7}	0
	Inh (92)	3×10^{-7}–3×10^{-5}				
Dosinia elegans (2)	Exc (100)	3×10^{-5}	0	Exc (100)	3×10^{-7}–1×10^{-6}	0
	Inh (100)	1×10^{-6}–3×10^{-5}				
Myidae						
Mya arenaria (5)	Exc (100)	3×10^{-9}–3×10^{-8}	0	Exc (100)	1×10^{-9}–3×10^{-8}	0
Pholadidae						
Cyrtopleura costata (6)	Exc (100)	1×10^{-9}–3×10^{-8}	0	Exc (100)	1×10^{-9}–1×10^{-8}	0
	Inh (100)	1×10^{-9}–1×10^{-7}		Inh (67)	3×10^{-9}–3×10^{-8}	

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* The taxonomy follows that outlined in Moore, 1969.

† Number of preparations failing to respond to any dose of agonist.

TABLE II

Summary of FMRFamide and 5HT dose-response profiles (DRPs) and derivation of species response indices (SRIs).*

SUBCLASS	FMRFamide				5HT			
	DRP categories				DRP categories			
Species (Number tested)	Exc	Comp	Inh	SRI	Exc	Comp	Inh	SRI
PTERIOMORPHIA								
Anadara lienosa floridana (2)	2	—	—	1e	2	—	—	1e
Anadara tuberculosa (9)	9	—	—	1e	6	3	—	2e
***Anadara ovalis* (7)	—	7	—	1c	—	7	—	1c
Noetia ponderosa (7)	—	6	1	2i	7	—	—	1e
Mytilus edulis (7)	7	—	—	1e	7	—	—	1e
Mytella guyanensis (6)	4	2	—	2e	6	—	—	1e
Brachidontes recurvus (4)	4	—	—	1e	2	2	—	2e
Modiolus squamosus (7)	7	—	—	1e	7	—	—	1e
Geukensia demissa demissa (23)	23	—	—	1e	—	14	9	2i
Geukensia demissa granosissima (8)	8	—	—	1e	—	2	6	2i
Atrina rigida (10)	6	4	—	2e	10	—	—	1e
†*Argopecten irradians* (8)	7	—	—	1e	8	—	—	1e
†*Lima scabra* (8)	7	—	—	1e	8	—	—	1e
†*Crassostrea virginica* (31)	2	11	8	3i	31	—	—	1e
†*Ostrea palmula* (7)	—	—	5	1i	7	—	—	1e
PALEOHETERODONTA								
Lampsilis claibornensis (21)	—	—	21	1i	3	18	—	2e
Lampsilis ovata ventricosa (8)	1	4	3	3i	8	—	—	1e
Lampsilis teres (8)	8	—	—	1e	8	—	—	1e
Ligumia recta (8)	8	—	—	1e	4	4	—	2e
Ligumia subrostrata (9)	1	4	4	3i	9	—	—	1e
Villosa villosa (12)	12	—	—	1e	2	10	—	2e
Villosa lienosa (6)	6	—	—	1e	1	5	—	2e

***Elliptio icterina* (5)	—	5	—	1c	2	3	—	2e
Anodonta peggyae (4)	4	—	—	1e	4	—	—	1e
Anodonta cataracta (3)	3	—	—	1e	3	—	—	1e
Quincuncina infucata (5)	1	4	—	2e	5	—	—	1e
HETERODONTA								
Chama pellucida (9)	6	3	—	2e	9	—	—	1e
Pseudochama exogyra (3)	—	1	2	2i	3	—	—	1e
Dinocardium robustum (9)	9	—	—	1e	9	—	—	1e
***Trachycardium egmontianum* (10)	—	3	7	2i	3	7	—	2e
***Rangia cuneata* (21)	2	19	—	2e	—	21	—	1c
Spisula solidissima (10)	10	—	—	1e	10	—	—	1e
Tresus nuttalli (11)	5	6	—	2e	11	—	—	1e
Ensis directus (5)	1	4	—	2e	5	—	—	1e
Semele decisa (3)	3	—	—	1e	3	—	—	1e
Semele rupicola (4)	1	2	1	3c	4	—	—	1e
Tagelus plebeius (7)	7	—	—	1e	7	—	—	1e
Polymesoda caroliniana (4)	4	—	—	1e	4	—	—	1e
***Corbicula manilensis* (12)	3	9	—	2e	3	9	—	2e
Mercenaria mercenaria (8)	8	—	—	1e	8	—	—	1e
Mercenaria campechiensis (10)	10	—	—	1e	10	—	—	1e
Chione cancellata (10)	10	—	—	1e	10	—	—	1e
Protothaca asperrima (7)	—	6	1	2i	7	—	—	1e
Tivela stultorum (7)	7	—	—	1e	5	2	—	2e
***Macrocallista nimbosa* (26)	24	2	—	2e	19	7	—	2e
Saxidomus nuttalli (8)	8	—	—	1e	8	—	—	1e
Dosinia discus (26)	2	21	3	3i	26	—	—	1e
Dosinia elegans (2)	—	2	—	1c	2	—	—	1e
Mya arenaria (5)	5	—	—	1e	5	—	—	1e
***Cyrtopleura costata* (5)	—	5	—	1c	2	3	—	2e

* DRPs can be excitatory (Exc), complex (Comp) or inhibitory (Inh).
** Species with complex DRPs to both FMRFamide and 5HT.
† Species that have one or more preparations failing to respond to FMRFamide.

TABLE III

The taxonomic distribution of species response indices (SRIs).

Agonist	Subclass	Species/SRI category 1e	2e	3e	1c	3c	3i	2i	1i
FMRFamide	Pteriomorphia	9	2	0	1	0	1	1	1
	Paleoheterodonta	6	1	0	1	0	2	0	1
	Heterodonta	1	6	0	2	1	1	3	0
	Totals	26	9	0	4	1	4	4	2
	(% of all species tested)	(52)	(18)	(0)	(8)	(2)	(8)	(8)	(4)
5HT	Pteriomorphia	10	2	0	1	0	0	2	0
	Paleoheterodonta	6	5	0	0	0	0	0	0
	Heterodonta	18	5	0	1	0	0	0	0
	Totals	34	12	0	2	0	0	2	0
	(% of all species tested)	(68)	(24)	(0)	(4)	(0)	(0)	(4)	(0)

(*Elliptio icterina, Trachycardium egmontianum,* and *Cyrtopleura costata*) have markedly different responses to the two agents. The complementarity of the approaches is most evident in the case of *Rangia cuneata*, however. The species responses are similarly positioned in the two mean response plots (Fig. 7), indicating that the *Rangia* hearts were excited and inhibited by about the same proportions of FMRFamide and 5HT doses. The SRIs indicate that, although inhibition was not the predominant effect, most *Rangia* preparations were inhibited by both agents (Table II). However, the species response curves differ in shape (Fig. 6); *i.e.*, 5HT inhibited only at relatively high doses, whereas FMRFamide inhibited at every dose tested. Thus, the *Rangia* hearts were similarly sensitive to 5HT inhibition but varied in their sensitivities to FMRFamide inhibition.

Pharmacological comparisons

Although both FMRFamide and 5HT were predominantly cardioexcitatory in over half of the species surveyed (SRI = 1e and 2e; Table III), neither agonist was exclusively so. Both inhibited some hearts at some doses.

The responses to FMRFamide were more variable than those to 5HT at all levels. First, a greater proportion of the FMRFamide species responses were moderately or highly diverse (SRI = 2 or 3; Table III), indicating greater variability of a single response from preparation to preparation within species. Second, there were more categories of FMRFamide SRIs and they had a broader species distribution (Table III), suggesting that the interspecific diversity of FMRFamide effects is also greater than that of 5HT effects. The points are more scattered in the FMRFamide mean response plot (Fig. 4), further supporting this conclusion.

As a corollary, the species responses to FMRFamide were less uniformly excitatory than those to 5HT. More FMRFamide species responses had inhibitory components, and inhibition was more frequently predominant (Table III). In addition, fewer points lie on the X-axis and more lie on, or close to, the Y-axis in the FMRFamide mean response plot (Fig. 4). If, in the two plots, we compare the points lying on the X-axes (N = 28 for FMRFamide and 35 for 5HT), the mean is lower for FMRFamide than for 5HT (arrowhead below each graph). Thus, on

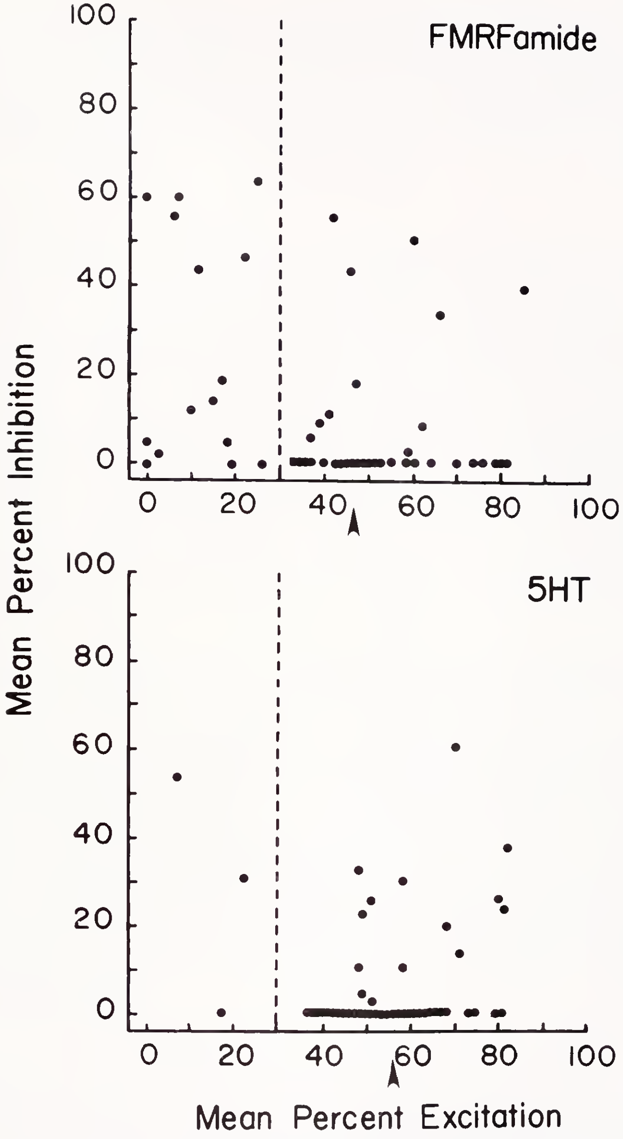
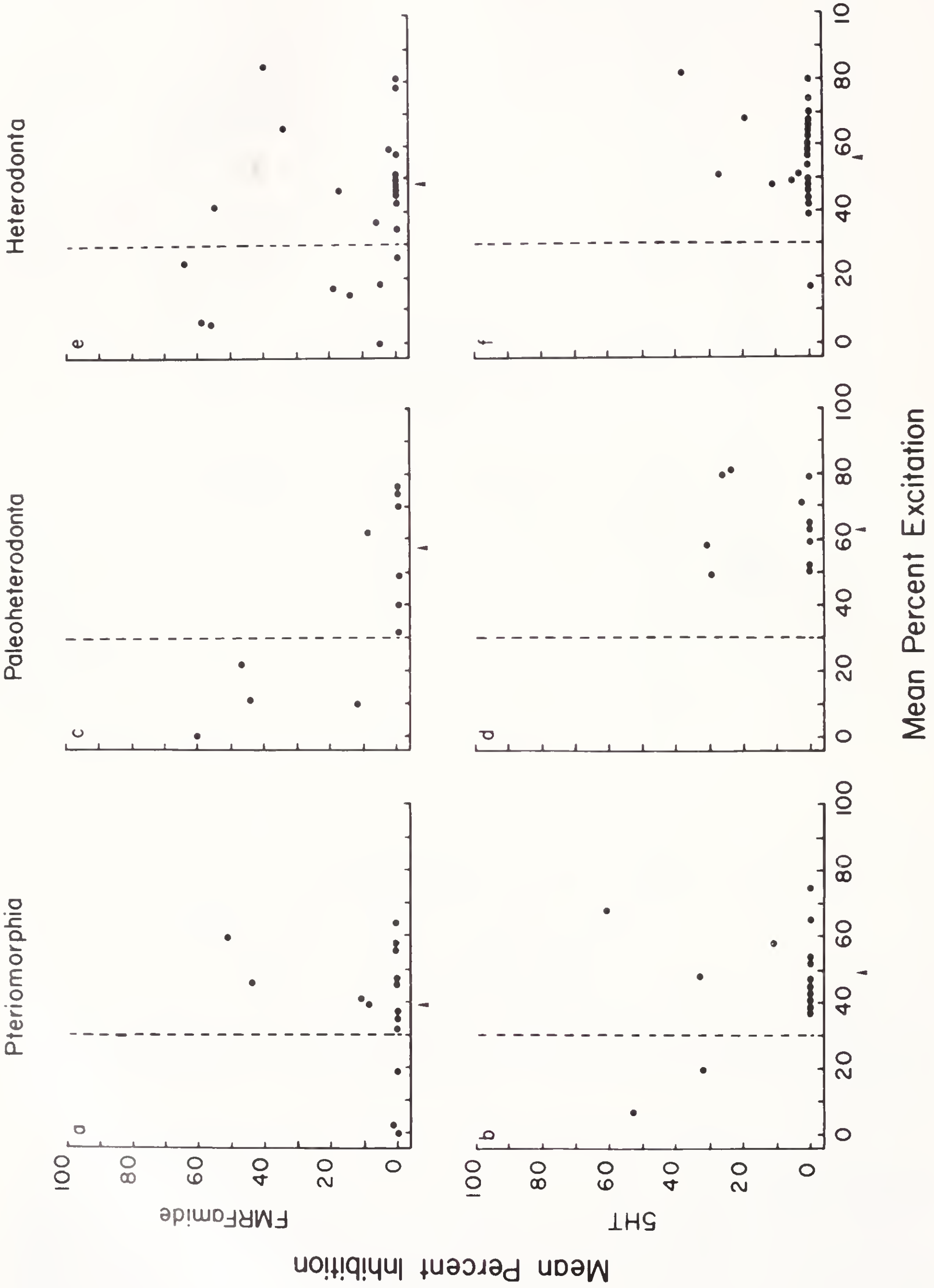


FIGURE 4. Composite mean response plots for FMRFamide and 5HT. The mean percentage of inhibitory doses is plotted as a function of the mean percentage of excitatory doses for each species. The arrowhead below each graph indicates the average mean percentage of excitatory doses for those species lying on the abscissa.

the average, fewer doses of FMRFamide were excitatory, though the difference in potency is small.

There was no consistent relationship between the responses to FMRFamide and those to 5HT. Fewer than half of the species surveyed had qualitatively similar responses to the two agonists. Of these, 20 species were solely excited by both compounds (Tables I and II); but seven of them were represented by only a few preparations. Seven species had FMRFamide and 5HT responses with both exci-


Pteriomorphia
Paleoheterodonta
Heterodonta
a
b
c
d
e
f
FMRFamide
5HT
Mean Percent Inhibition
Mean Percent Excitation
0
20
40
60
80
100

tatory and inhibitory components, but, as described above, the responses were qualitatively similar in only three of them (Table II; Figs. 6 and 7).

In conclusion, the comparisons between the responses to FMRFamide and 5HT show that neither agent is exclusively cardioexcitatory, that the effects of FMRFamide are more often inhibitory than those of 5HT, and that the variation in the responses to the two agonists is not in parallel.

Taxonomic considerations: subclasses

Phylogenetic patterns in the pharmacologies of FMRFamide and 5HT are evident at the level of subclass. However, some of the general conclusions outlined above, based on an overall comparison of the two agonists, begin to break down when the comparisons are restricted to members of a specific subclass. In particular, although the general conclusion that FMRFamide effects are less uniform and more inhibitory than those of 5HT holds for the Paleoheterodonta and Heterodonta, the relationship is exactly opposite in the Pteriomorphia. Details follow.

In both the Paleoheterodonta and Heterodonta, the FMRFamide SRIs were more disparate than those of 5HT, had a broader species distribution, and had more significant inhibitory components (Table III). Furthermore, the points were more scattered in the FMRFamide mean response plots, indicating that members of these two subclasses varied more in their average sensitivities to FMRFamide than to 5HT (Fig. 5).

In contrast, both agonists were exclusively cardioexcitatory in about two-thirds of the pteriomorphs surveyed, and both inhibited about the same proportions of species (Table III). However, the points were more scattered in the pteriomorph mean response plot for 5HT, indicating that pteriomorph hearts varied more in their average sensitivities to 5HT than to FMRFamide. Moreover, two of the three species responses lying to the left of $X = 30$ in the composite 5HT plot were pteriomorphs, while only three of fifteen species lying in this region of the FMRFamide graph belonged to this subclass (Figs. 4 and 5).

In summary, and as we concluded above, 5HT is rarely inhibitory or even weakly excitatory. But when such an effect does occur, it is most likely to be on a pteriomorph heart. Conversely, FMRFamide is more likely than 5HT to be inhibitory or weakly excitatory, and these effects usually occur in paleoheterodont or heterodont preparations.

Taxonomic considerations: families

Phylogenetic patterns in the pharmacologies of FMRFamide and 5HT are also discernible at the family level. The effects of FMRFamide and 5HT can be remarkably uniform within particular families, though there are usually some exceptional species. Other families have characteristically diverse species responses, especially to FMRFamide. The response characteristics of some families reflect the features of their subclass, while a few are unique. Four families illustrate this assortment of relationships; they are examined below.

Unionidae. Most extant paleoheterodonts, and all of those included in this survey, are members of the family Unionidae. The effects of FMRFamide on unionid

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FIGURE 5. Mean response plots for FMRFamide and 5HT on pteriomorph (a, b), paleoheterodont (c, d) and heterodont (e, f) hearts. The mean percentage of inhibitory doses is plotted as a function of the mean percentage of excitatory doses. The arrowhead below each graph indicates the average mean percentage of excitatory doses for those species lying on the abscissa.

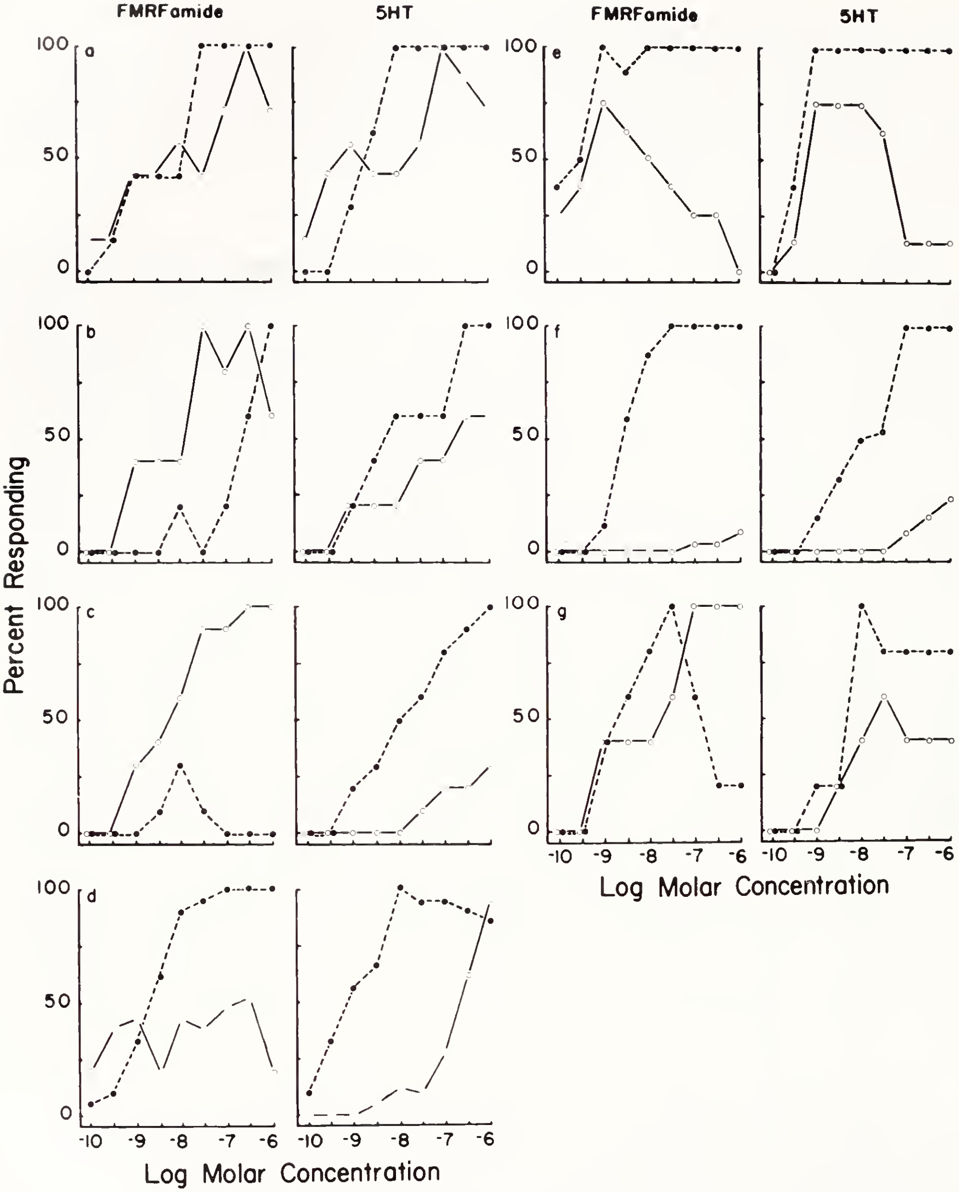

FIGURE 6. Species response curves for seven species with complex DRPs to FMRFamide and 5HT. These curves show the percentage of preparations excited (dashed line) and inhibited (solid line) by each dose of agonist between 10^{-10} *M* and 10^{-6} *M*. a) *Anadara ovalis.* b) *Elliptio icterina.* c) *Trachycardium egmontianum.* d) *Rangia cuneata.* e) *Corbicula manilensis.* f) *Macrocallista nimbosa.* g) *Cyrtopleura costata.*

hearts were characteristically diverse, even within single genera (*e.g., Lampsilis* and *Ligumia*) (Tables I, II and III). Inhibition was common, and was often a significant or predominant component of the response (*e.g., Lampsilis claibornensis*). Yet other species (*e.g., Lampsilis teres*) were only excited by the peptide.

In contrast, all unionid preparations were excited by relatively low doses of

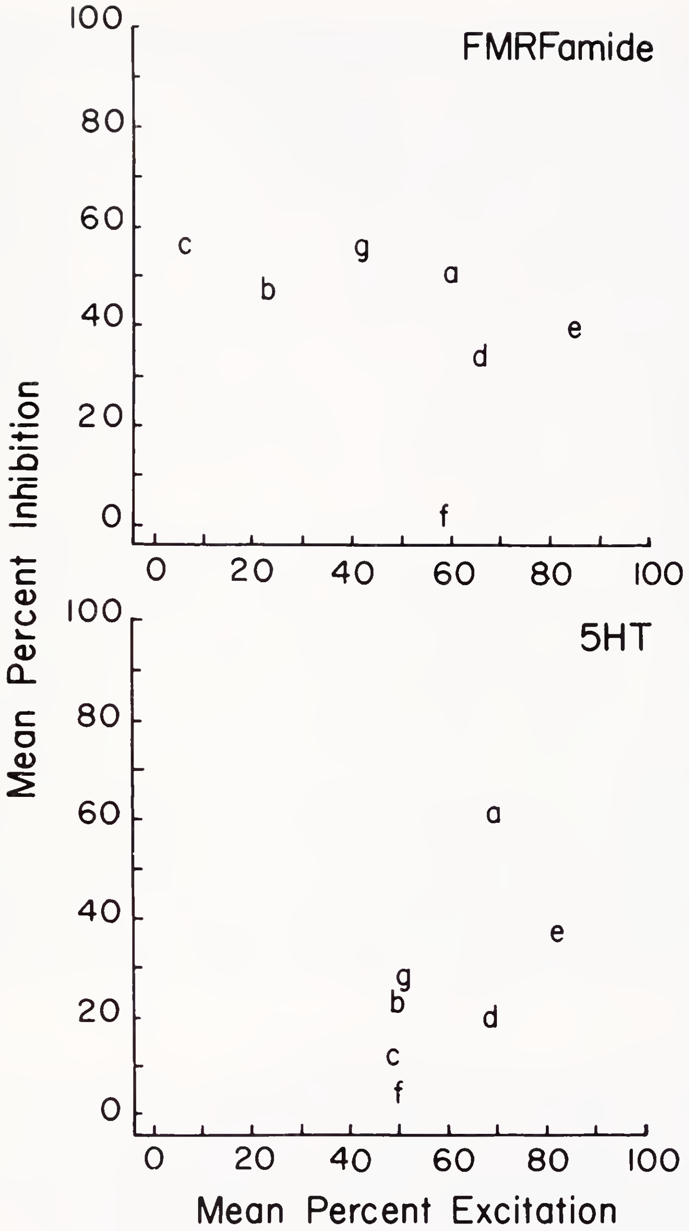

FIGURE 7. Mean response plots for seven species with complex DRPs to both FMRFamide and 5HT. The mean percentage of inhibitory doses is plotted as a function of the mean percentage of excitatory doses. Each species response is designated by a letter corresponding to that used in Figure 6. a) *Anadara ovalis.* b) *Elliptio icterina.* c) *Trachycardium egmontianum.* d) *Rangia cuneata.* e) *Corbicula manilensis.* f) *Macrocallista nimbosa.* g) *Cyrtopleura costata.*

5HT. A slowly developing inhibition appeared at higher doses in some preparations from about half of these species (Tables I, II and III). Thus, in the family Unionidae (subclass Paleoheterodonta), as in the subclass Heterodonta, the effects of 5HT are more uniform and less inhibitory than those of FMRFamide.

Veneridae. The responses of venerid hearts were not characteristic of their subclass, the Heterodonta: both FMRFamide and 5HT were overwhelmingly cardioexcitatory in this family (Tables I and II). The thresholds for excitation by both agonists were usually low (but see *Dosinia discus* and *D. elegans*; Table I). Cardioinhibition was rare and, with a single exception (FMRFamide inhibition of *Protothaca asperrima* hearts; Table I), appeared only at high doses.

Mytilidae. The singularity of pteriomorphian pharmacology is primarily a reflection of the species responses of the Mytilidae. FMRFamide was always cardioexcitatory in this family. Preparations from only one species (*Mytella guyanensis*) were ever inhibited by the peptide (Tables I and II), and the effect was transient, preceding a sustained excitation. In comparison, the effects of 5HT were more varied and often inhibitory. Inhibition appeared only at high doses in most species, but was the predominant effect in the two subspecies of *Geukensia. Geukensia* hearts were unique among all of the bivalve ventricles surveyed in this characteristic (Tables I and II). This uniqueness is also reflected in the composite 5HT mean response plot (Fig. 4): the *Geukensia* responses are the only points lying to the left of X = 30 and above the X-axis.

Ostreidae. Oyster hearts were notably insensitive to FMRFamide. None of the *Ostrea* and less than one-third of the *Crassostrea* ventricles responded to the highest FMRFamide concentrations routinely tested (1×10^{-6} *M*); and nearly one-third of the hearts from both species failed to respond to the highest doses ever tested ($1\text{–}3 \times 10^{-5}$ *M*; Table I). Only two other species (*i.e., Lima scabra* and *Argopecten irradians*), both belonging to the same order as the oysters (Pteroida), contained any preparations that failed to respond to such high doses of FMRFamide (Tables I and II). Nonetheless, compared to the oysters, these hearts were relatively responsive to the peptide (Table I). Thus, the oyster hearts are clustered alone at the origin of the FMRFamide mean response plot (Fig. 4), illustrating both the uniqueness of the effects and the efficacy of the analytical technique.

Ostreid responses to FMRFamide, when they occurred, were small and transient. *Ostrea* ventricles were only inhibited by the peptide, but *Crassostrea* ventricles were variously affected (Table II). Considering the small number of *Ostrea* hearts surveyed (N = 7, compared to 31 for *Crassostrea*), and the diversity of the *Crassostrea* species response, we suppose that further sampling of *Ostrea* preparations would also have revealed a greater diversity of effects.

DISCUSSION

We have surveyed the effects of FMRFamide and 5HT on the mechanical activity of more than 450 ventricles from 50 species of bivalved molluscs; this is about 1% of the class Bivalvia. Considering that the objects of study were homologous organs, often from closely related species, the responses were strikingly diverse, varying qualitatively with dose as well as species. Since the usual pharmacological analyses of dose-response relationships are not well designed to deal with these kinds of variation, we developed some new approaches that allowed us to express, succinctly, the response of a preparation or species. Comparisons between drugs and taxa were made possible by the application of these techniques, and three major generalities emerged.

First, although most species responses to FMRFamide and 5HT are predominantly excitatory, both compounds inhibit the hearts of some species at some doses. Clearly, FMRFamide is not a general excitor of molluscan muscle and nerve (*e.g.,* Greenberg and Price, 1979). Ironically, the notion that it is probably arose because the first, and most thoroughly, studied bivalve hearts were those of the atypical family Veneridae.

Second, the actions of FMRFamide and 5HT do not vary in parallel; thus FMRFamide is also not a serotonomimetic agent. Nevertheless, there are some systematic relationships between the two sets of responses which are evident at the level of subclass. In particular, FMRFamide is more likely than 5HT to be inhibitory or weakly excitatory, and these effects appear most commonly in the Paleoheterodonta and Heterodonta. In contrast, 5HT is only rarely inhibitory or even weakly excitatory; and such effects are most likely to occur in the subclass Pteriomorphia. This dichotomy between pteriomorph responses and those of the paleoheterodonts and heterodonts was not unexpected since the Paleoheterodonta and Heterodonta are more closely related to each other than either is to the Pteriomorphia (Purchon, 1978). Moreover, the pharmacological dissimilarity is in conformity with other physiological differences between pteriomorph and heterodont hearts, including the ionic bases of excitability (Deaton and Greenberg, 1980), the levels and forms of cholinesterase (Roop and Greenberg, 1976; Greenberg *et al.*, 1980), and the sodium-calcium exchange across the sarcolemma (Plumb and Koch, 1979).

Third, the responses to either FMRFamide or 5HT can be strikingly uniform in some bivalve families, and be characteristically diverse in others. Thus, although systematics and pharmacology are correlated, the effects of FMRFamide and 5HT are neither consistent nor reliable characters of bivalve families. The actions of cholinergic drugs on bivalve hearts are also loosely correlated with taxonomy (Greenberg, 1965; Greenberg *et al.*, 1980), and we suppose that, were it systematically tested, such a general correlation would be a feature of all drug-organ interactions.

Finally, several mechanisms of action undoubtedly underlie the diverse effects of FMRFamide and 5HT observed in this survey. These mechanisms remain to be investigated, however, and speculations about them, based on intensive studies of the actions of these or other drugs on the hearts of particular bivalve species (*e.g.*, Higgins *et al.*, 1978; Elliott, 1980), would probably prove to be premature. Nevertheless, this survey has provided a set of model systems which are being exploited in such investigations (*e.g.*, Painter, 1982).

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AN INDEX OF AGE WHEN BIRTHDATE IS UNKNOWN IN *APLYSIA CALIFORNICA*: SHELL SIZE AND GROWTH IN LONG-TERM MARICULTURED ANIMALS

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Abstract

The purpose of this study was to determine the age of postmetamorphic *Aplysia* when the birthdate was not known. The study is based upon the long-term growth and maintenance of *Aplysia californica* kept in artificial sea water up to 235 days. Body and organ weights and organ size were measured. The most reliable measure of growth and age was the size of the internalized shell.

The rate of shell growth was determined, and on the average it was 0.23 mm/day throughout post-metamorphic life. A means of determining age using shell size is given. The results reported here are used to determine the age of *Aplysia* in which age-dependent changes in behavior and in anatomical and physiological properties of single nerve cells have been found.

Introduction

Aplysia californica is a marine gastropod mollusc with a life span of approximately one year (Eales, 1921; Kandel, 1976; Audesirk, 1979). Laboratory studies revealed that *Aplysia* pass through both an embryonic and a larval stage before developing into the adult form: after oviposition, fertilized eggs undergo embryonic development of 8–10 days and then, as is typical for marine gastropods, pass through a veliger larval stage for 30–34 days; metamorphosis lasts for 2 to 3 days (Kriegstein *et al.*, 1974; Kriegstein, 1977; Switzer-Dunlap and Hadfield, 1977). Immediately after metamorphosis occurs, a miniaturized version of the larger and older post-metamorphic animal appears. *Aplysia* can be cultured in the laboratory from oviposited eggs to sexually mature animals in natural sea water (Kriegstein *et al.*, 1974; Switzer-Dunlap and Hadfield, 1977), but as yet not in artificial sea water (Kriegstein, personal communication; Peretz, unpublished observations). At present there is no reliable means of determining age of *Aplysia* other than body weight without knowing the birthdate. Body weight is subject to considerable variation (Audesirk, 1979). Most studies on the *Aplysia* nervous system, which contains identifiable neurons, have been done on post-metamorphic animals (for review see Kandel, 1976) of unknown age.

Peretz and Lukowiak (1975) found physiological and behavioral differences between young and sexually mature *Aplysia*, both of which were post-metamorphic groups. Their results prompted further studies of age-dependent neurophysiological, neuroanatomical, and behavioral changes in *Aplysia*. Recent studies showed that the *Aplysia* nervous system with its identifiable neurons is a useful preparation to study the life history of single neurons (Papka *et al.*, 1981; Rattan and Peretz, 1981; Peretz *et al.*, 1982). Characteristics of aging in *Aplysia* neurons show a

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striking resemblance to those in mammalian neurons (Papka *et al.*, 1981). It was necessary to determine the age of post-metamorphic animals whose birthdate was not known with reliability greater than that obtained using body weight. The literature dealing with molluscan growth and allometry is extensive with respect to animals studied in the field (Wilbur and Owen, 1964; see also Comfort, 1957; Huxley, 1972). Laboratory studies of the growth of gastropod molluscs are less extensive (Carefoot, 1967; Kriegstein *et al.*, 1974; Kempf and Willows, 1977; Kriegstein, 1977; Switzer-Dunlap and Hadfield, 1977). The findings reported here provide a means of determining age of post-metamorphic *Aplysia.*

MATERIALS AND METHODS

Post-metamorphic *Aplysia californica* obtained from Pacific Biomarine, Inc. (Venice, California) ranged in weight from 0.1 g to 1100 g. In this study, begun in January 1979, body weight and organ measurements were made in 378 animals. Upon arrival, animals were weighed and tagged for identification and then placed in holding tanks. Plastic tags which were numbered and color-coded were attached to the posterior portion of one of the parapodia by surgical thread. A loop of thread was used so that it did not interfere with growth of the parapodium; thread tied too tightly to the parapodium eventually cut through the tissue and fell off. Very young animals, kept in perforated refrigerator containers in the holding tanks, were tagged after attaining a body weight of 50 g. Each animal was weighed once a week. Damp weight of animals was subject to less variation on repeated measures than attempting to determine the "maximum stretched" length by anesthetizing animals with an injection of $MgCl_2$. It was felt that the possible cumulative effects of repeated anesthetization with $MgCl_2$ on subsequent neurophysiological and behavioral experiments should be avoided. When an animal was sacrificed, the reproductive tract, radula, gill, and shell were weighed. Maximum shell diameter was also measured.

All animals in this study were well past metamorphosis. In post-metamorphic animals, the tissue constituting the mantle flap covers the shell (see Kriegstein, 1977); this structure strengthened by the shell is located in the pallial cavity overlaying and protecting the gill. The shell was removed by cutting away the mantle tissue and gently removing it from its attachment at the base of the mantle. The shell contains bands analogous to the rings seen on shells of bivalve molluscs. The distinctness of the bands was highly variable from animal to animal and thus could not be counted nor used as an index of growth. Also, growth rings on the shells of certain species of bivalves are affected by environmental conditions and thus are not solely expressions of growth and age (Jones, 1981); this may hold also for the *Aplysia* shell. The method used to measure the maximum shell diameter was similar to that described by Wilbur and Owen (1964) for bivalves. The diameter is the perpendicular distance from the umbo to the line drawn tangent to the point of maximum curvature (inset Fig. 5).

Animals are kept in artificial sea water (Instant Ocean, Eastlake, Ohio). The 450 gallon recirculating system consists of three 50 and two 10 gallon holding tanks, two reservoirs of 145 gallons each and a filter; metal-free pumps (March Pumps, Glenview, Ill.) are used to circulate the sea water. The temperature is held at 16 ± 1°C throughout the year by passing it through plastic tubing submerged in a 55 gallon container of chilled tap water. The specific gravity is maintained at 1.023 to 1.024 by adding tap water to the reservoir when necessary. The sea water is passed through a filter composed of a bottom layer of activated charcoal, 5 inches thick, which removes finely suspended material; above it a layer of crushed oyster shell, 6 inches thick, which is used to buffer the sea water at pH 7.8 ± 0.1; and above that a porous polyurethane mat prevents passage of larger pieces of fecal matter and detritus. The system is cleaned once a week.

Animals are fed five times per week: the smallest animals, up to 10 g are fed fresh *Plocamium* and those 10 g and over are fed dried red laver, *Porphyra*, (Vega Trading Co., New York) twice per week and fresh *Lactuca sativa longifolia*, romaine lettuce, three times per week.

RESULTS

The objective of this study was to find a convenient and reliable index of growth in post-metamorphic animals and consequently to determine their age under laboratory conditions—that is, in artificial sea water, invariant temperature, fixed photoperiod of 12:12, and dried seaweed and soil-grown lettuce. As will be seen below, rate of body weight increase was found to be similar to that of animals raised under conditions more closely resembling those in the field. The rate of increase was dependent on size rendering it an unreliable index of age. Also, considerable weight change results from egg laying.

General observations

Animals that consistently gained weight, regularly ambulated in the holding tanks, and laid eggs after attaining sexual maturity survived the longest. Of the 236 animals whose weights were recorded for more than two weeks, 84 percent gained weight.

Loss of weight for three consecutive weeks usually signalled impending death. Loss of weight also followed egg laying (Fig. 1). In sexually mature animals (100–250 g) the weight was regained. This is consistent with the observation of Audesirk (1979). In contrast, in old animals (500 g and greater) weight loss was not regained (Fig. 1). Regaining of weight after egg laying appears to be age-dependent.

Of the 378 animals studied, 1.6 percent died within two weeks of their arrival; the mortality rate of the animals kept beyond two weeks was only 19 percent. Deaths were not correlated with a particular season. Animals received in winter survived well into spring; those received in the summer survived beyond winter. This observation suggests that death of *Aplysia* is not a seasonal event, and that their survival is dependent upon internal factors rather than environmental factors.

Growth as measured by body weight

Animals were weighed once a week, and growth rates as expressed by increases in body weight were measured (Fig. 2A). Young animals (juveniles) whose average weight upon arrival was 1.7 g were cultured in the holding tanks for 105 days at which time they had grown to an average weight of 170 g. Growth follows a sigmoid-like curve. As is typical for growth curves, Figure 2A shows an initial "lag" phase. Body weight increases approximately 0.28 g/day. During "log" phase body weight doubles every 14 days from the 28th day, the rate increases to 2.1 g/day. Body weight doubles every 7 to 10 days up to 100 g and then approximately once every 25 days.

When larger animals (mature) are cultured and body weight measured over a comparable period of time, the growth rate is different than that in Figure 2 (curve A). Figure 2 (curve B) shows that a lag phase is absent and the growth is

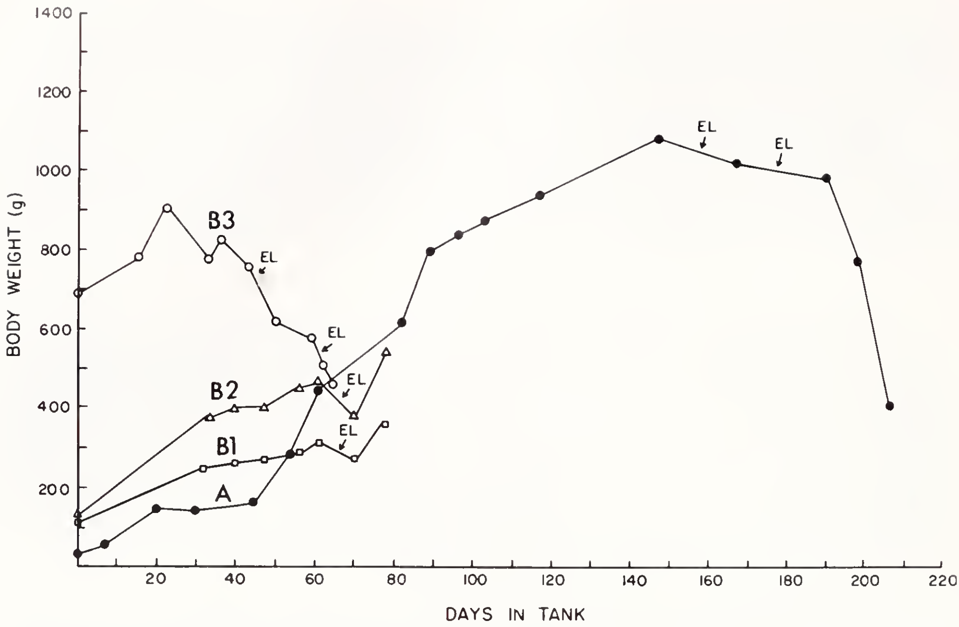

FIGURE 1. An animal cultured from an initial weight of 12 g, arrival date 26 January, 1979, to a maximum weight of 1090 g (curve A). Egg laying (EL) occurred 160 days after arrival. Note that animal gained 150 g within one week and lost 400 g within one week. The variability in weight changes seen here is typical for animals in this study. Three animals from the same shipment showing the effect of egglaying (EL) on body weight (curve B1–3). The upper curve is from an old animal who did not recover from weight loss. The two lower curves are of mature animals approximately 70 days younger than the old animal, which show recovery of body weight. Spontaneous egg laying occurred in the holding tanks throughout the calendar year rather than during a specific egg laying season.

more linear. The growth rate of 3.6 g/day is greater than that for younger animals. Animals 500 g and larger have a growth rate of 7.6 g/day. Table I shows that growth rates vary considerably from weight group to weight group, with the coefficient of variation ranging from 13 to 57 percent and averaging 30 percent. The rate of increase of body weight appears dependent upon the age of the animal. These results in addition to those in Figure 1 show that body weight is not a reliable index of age.

With the kind offer from Dr. J. Vallee (Pacific Biomarine, Inc.) of a growth curve of animals cultured from eggs, we were able to compare growth in the holding tanks with animals grown in natural sea water (Fig. 2B). Animals grown from eggs were cultured at 18 to 20°C and fed fresh seaweed *ad libitum*. The growth rate during "lag" phase appears to be 0.25 g/day. The growth rate during "log" phase is approximately 3 g/day which is comparable to that of growth in artificial sea water. In another study in which animals were raised from oviposited eggs in natural sea water at 22°C and fed regularly, the growth rate of young animals nearing sexual maturity was approximately 6 g/day (Kriegstein *et al.*, 1974; Kriegstein, personal communication). The difference between Kriegstein's results and ours probably is due to the difference in ambient temperature, 22°C *vs* 16°C. The difference between Vallee's results and those of Kriegstein could be explained by the difference in feeding regimen and possibly the difference in temperature.

During 2.5 years of measuring body weights seasonal variations in the growth

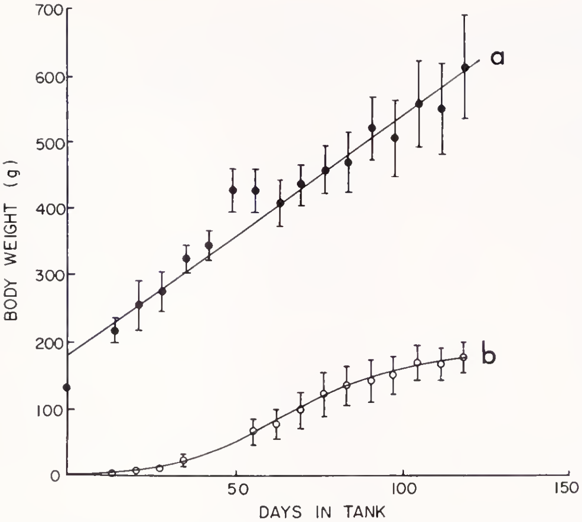

FIGURE 2(A). Growth curve of animals whose average initial weight was 1.7 g (curve A). The average body weight of the group (n = 10) is plotted against days cultured in tanks. The animals were raised from young to sexually mature animals. The equation which best relates body weight (w) to time (t) in the holding tanks is: $w = 3.4 - 0.8t + 0.5t^2 - 2.4 \times 10^{-4}t^3$. This equation only holds for animals up to 200 g. Growth curve of animals whose average initial weight was 132 g (curve B). The average body weight of the group (n = 8) is plotted against days cultured in tanks. The animals were raised from sexually mature to old animals. The growth rate is comparable to those animals of curve A and Figure 2B whose weights exceed 100 g. The equation which best relates body weight (w) to time (t) in the holding tanks for mature animals is: $w = 182 + 3.6t$. This equation only holds for animals above 100 g.

rate have not been observed. It is possible that seasonal effects disappear when animals acclimate to conditions in the holding tanks after two weeks.

Organ weight in relation to body weight

An allometric study was carried out between reproductive tract, radula, gill, and shell weights and body weight (Fig. 3). Body weight *vs* organ weight was plotted logarithmically, because allometric relationships obey the power law, *i.e.*, $y = ax^b$ (von Bertalanffy, 1960; Jones, 1981). (The equations for the best fit line are given in Figure 4.) Minimal change of shell and radula weights was observed up to a body weight of 50 g. The organ weights of animals above 50 g increase with considerable scatter. At a given body weight the coefficient of variation is on the average 49 percent for shell weight and 26 percent for radula weight. The relationship of gill and reproductive tract weights to body weight also displays considerable scatter. At a given body weight the average coefficient of variation of gill weight is 38 percent and 51 percent for reproductive tract weight. The use of any of the four organ weights would result in a less reliable estimate of age than using body weight alone.

Shell size in relation to body weight

A more consistent relationship was found between maximum shell diameter and body weight, plotted logarithmically (Fig. 4). The shell diameter increases with body weight throughout the post-metamorphic life of *Aplysia*. At a given body

TABLE I

Rate of body weight increase.

Weight range (g) during growth	grams/day Initial Weight 1 g	Initial Weight 120 g
1–10	0.57 ± 0.33 n = 15	—
10–50	1.0 ± 0.23 n = 12	—
50–120	3.3 ± 1.6 n = 9	—
120–500	4.2 ± 0.53 n = 11	4.3 ± 0.58 n = 4
120–1200	—	5.7 ± 1.5 n = 9
500–1200	5.3 ± 1.2 n = 4	7.4 ± 2.8 n = 9

weight shell diameters of recent arrivals, sacrificed within two weeks, and animals in the holding tanks for longer periods, up to 235 days, were the same. The coefficient of variation for the diameter at a given body weight does not vary more than 8 percent. Non-linear regression analysis of the data yielded a best fit curve given by the equation, $Y = 5.1 \times W^{0.33}$ ($n = 347$); where Y is the shell diameter and W is the body weight; the correlation coefficient is 0.99. The form of the

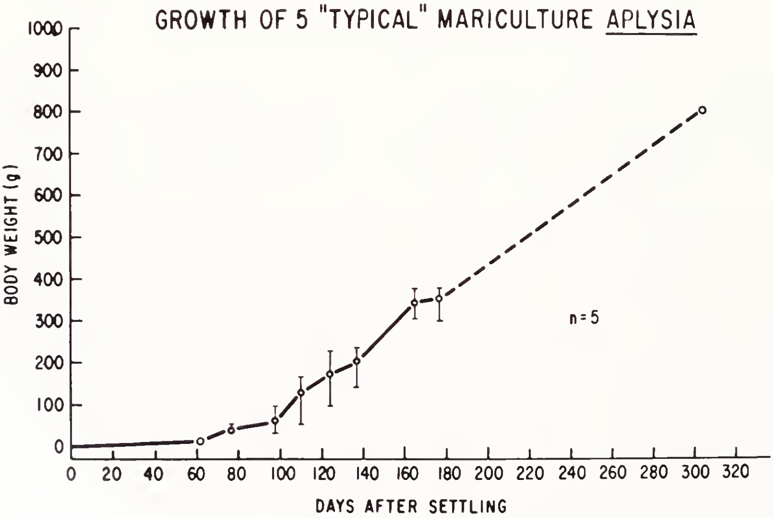

FIGURE 2(B). Growth curve of animals cultured from oviposited eggs in natural seawater at 20°C and fed fresh seaweed. Animals at 60 days after settling are equivalent to animals in (A) at zero time in tanks. Note the initial lag phase and subsequent log phase. The growth rates in A and B are comparable. (Modified from curve supplied by Dr. J. Vallee.)

TABLE II

Rate of shell growth (mm/day).

	1*	2*	3†	4‡	5*	6*	7*
	[DS]	[SS]	[DS]	[DS]	[SS]	[DS]	[DS]
Initial SD—mm	4.6 ± 0.4 (0.9 g)	4.6 ± 0.4 (0.9 g)	4.6 ± 0.4 (0.9 g)	25.1 ± 2.2 (100 g)	25.1 ± 2.2 (100 g)	25.1 ± 2.2 (100 g)	41.2 ± 3 (500 g)
Growth Interval	131 ± 32 days	100 ± 53 days	79 ± 26 days	51 ± 29 days	85 ± 27 days	90 ± 29 days	30 ± 13 days
Final SD—mm	30.1 ± 2.4 (220 g)	35.6 ± 4.7 (385 g)	15.8 ± 2.4 (30 g)	30.5 ± 2.4 (250 g)	41.2 ± 3.0 (500 g)	41.2 ± 3.0 (600 g)	53.3 ± 3.1 (1200 g)
Rate mm/day ± SE	0.24 ± 0.02 n = 10	0.28 ± 0.04 n = 8	0.13 ± 0.03 n = 7	0.14 ± 0.01 n = 9	0.21 ± 0.02 n = 17	0.21 ± 0.03 n = 11	0.21 ± 0.05 n = 8

Weighted average of columns 1, 2, 5, 6, and 7: 0.23 ± 0.02 mm/day, n = 54

* No significant difference between columns 1, 2, 5, 6, and 7, $P > 0.1$ (Mann-Whitney Test).
† Significant difference between columns 1 and 3; $P < 0.05$ (Mann-Whitney Test).
‡ Significant difference between columns 4 and 5; $P < 0.05$ (Mann-Whitney Test).
[DS] Animals from different groups.
[SS] Animals from same group.
Average body weight in parentheses.

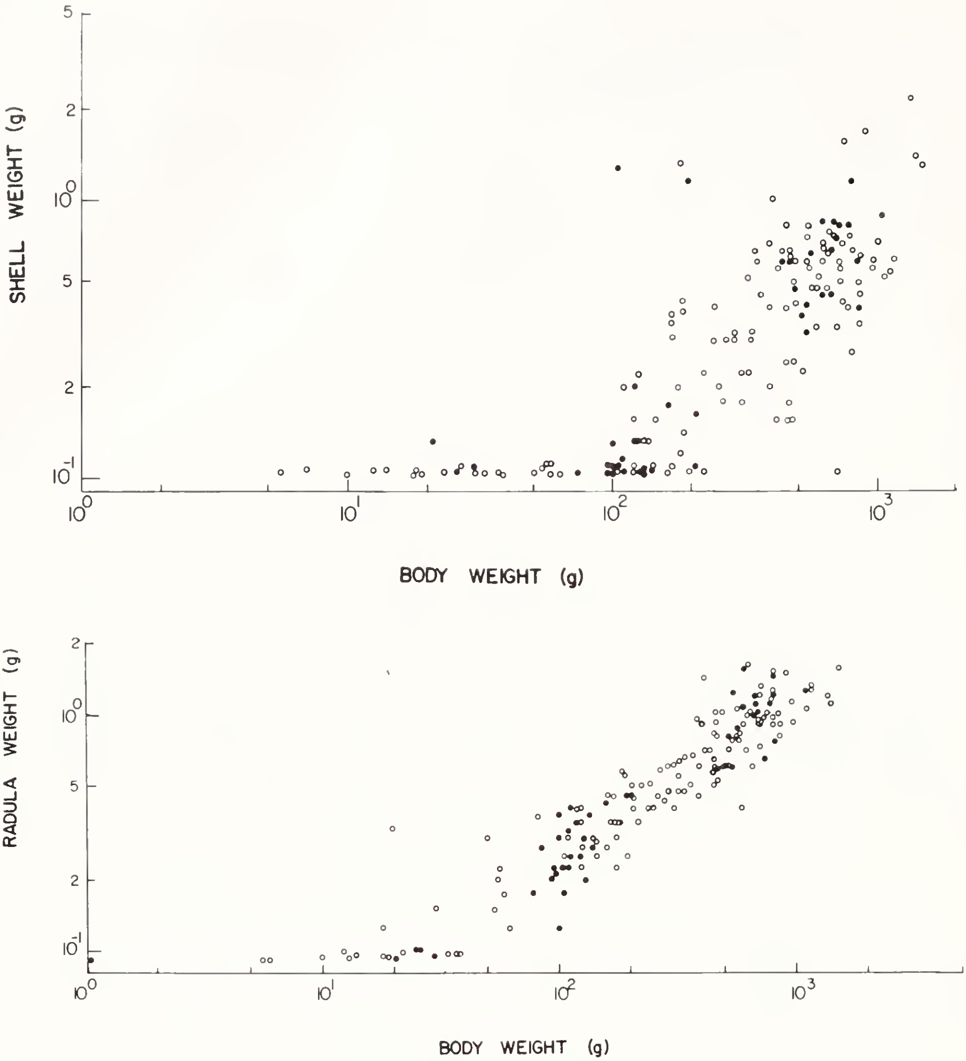

FIGURE 3(A), (B), (C), (D). Relationship between organ weights and body weights plotted logarithmically. Closed circles (●) indicate measurements taken from newly arrived animals, and open circles (○) are those from animals cultured for at least 30 days. Note that there is no difference between newly arrived and cultured animals. The equations for the best fit line for the allometric relationships is given below:

(A) Shell wt *vs* BW	(C) Gill wt *vs* BW
$Y = 3 \times 10^{-3}X^{0.78}$	$Y = 9 \times 10^{-3}X^{0.95}$
(B) Radula wt *vs* BW	(D) Reproductive tract wt *vs* BW
$Y = 8 \times 10^{-3}X^{0.74}$	$Y = 4 \times 10^{-3}X^{0.99}$

equation is the same as that for relating shell diameter to body weight in bivalve molluscs (Wilbur and Owen, 1964). Also Huxley (1972) has shown that organ size or length of a body part varies with the cube root of body weight (see also von Bertalanffy, 1960).

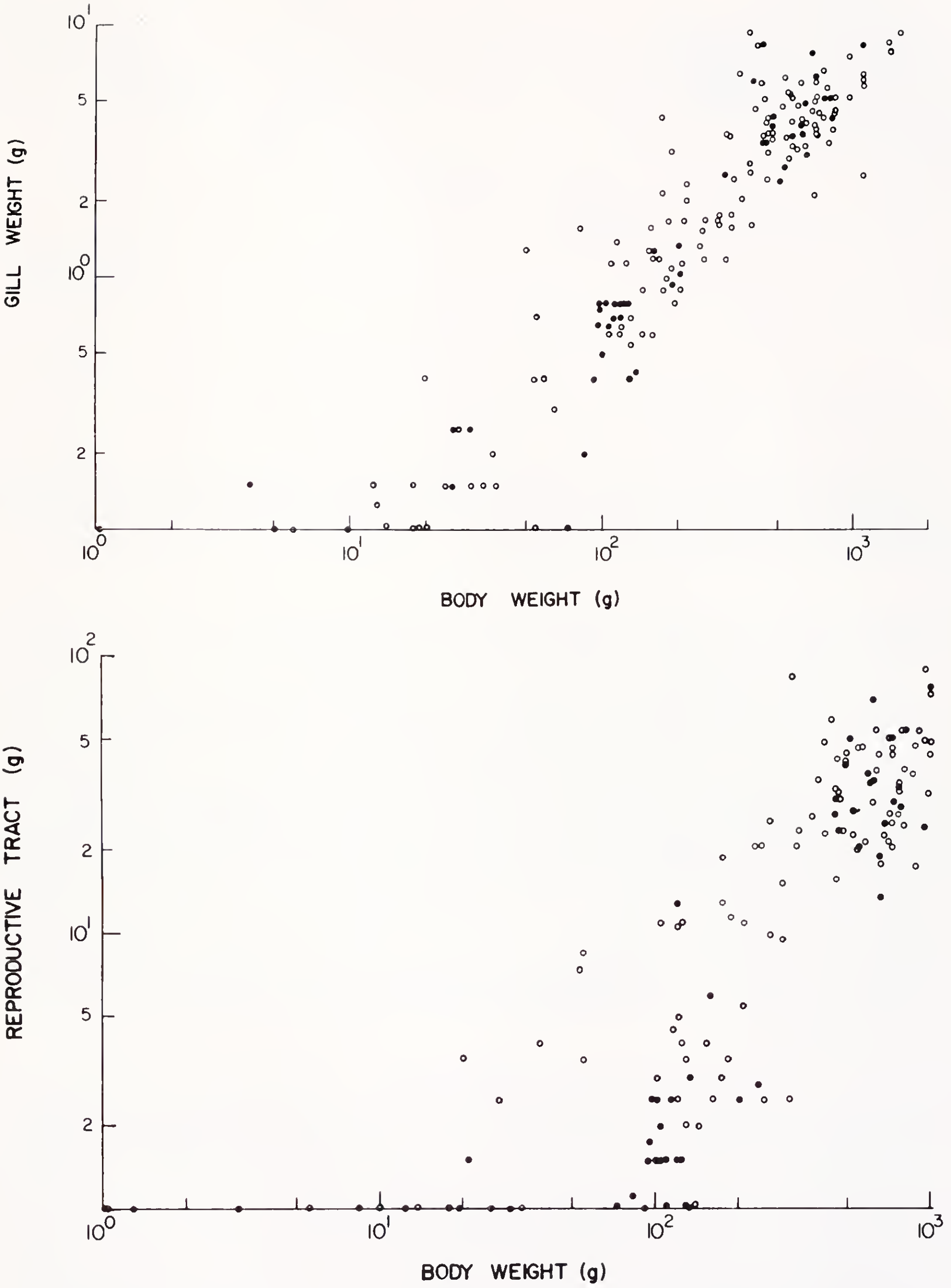

FIGURE 3. (Continued)

Rate of shell growth

We sought to determine the rate of shell growth and investigated the possibility that the rate of shell growth is the same in small and large animals, that is, in-

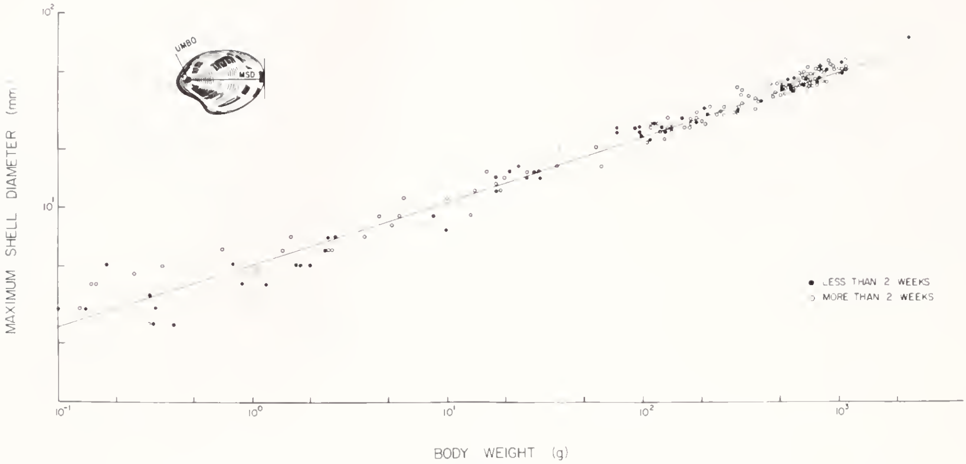

FIGURE 4. Relationship between shell diameter and body weight. Closed circles (●) indicate measurements taken from newly arrived animals, and open circles (○) are those from animals cultured for at least 30 days. Note that there is little scatter over the range of 0.5 g to 1250 g. The inset shows how the maximum shell diameter was measured.

dependent of body weight. This would be in marked contrast to rate of increase in body weight which is size dependent (Fig. 2, Table I).

The following procedure was carried out to determine the rate of shell growth. Initially, animals from the same shipment were used; subsequently, animals from different shipments were used (Table II). Pairs of animals were selected whose weights upon arrival were within 10 percent of each other; one was used as the reference animal and was sacrificed within two weeks of arrival and the other was kept in the tanks anywhere from 1 to 5 months and then sacrificed. The shell diameter of each animal was measured as shown in Figure 4. Table II shows the results of this study. There is no significant difference in the rate of shell growth in animals of different weights, $P > 0.1$ (Mann-Whitney Test, Table II; see also Fig. 5). The average shell growth was 0.23 ± 0.02 mm/day ($n = 54$). Animals which gain little or no weight had an average shell growth rate of 0.13 to 0.14 mm/day regardless of initial weight. There did not appear to be seasonal variations in shell growth.

DISCUSSION

Aplysia californica can be kept in healthy condition for most of their postmetamorphic life in artificial sea water as long as they are fed adequately. Under the conditions described in this paper animals grow, mature sexually, and lay eggs. The rate of growth in the holding tanks was comparable to that of animals kept under conditions more closely approximating those in the field. As a result of long-term maintenance in the holding tanks, weight gain and allometric measurements of organs were made to find a reliable index of age. Of the measurements made, the most reliable found was the rate of shell growth. The results are consistent with previous studies (Tessier, 1960; von Bertalanffy, 1960; Wilbur and Owen, 1964; Huxley, 1972, Audesrik, 1979).

Using the rate of shell growth obtained from matched pairs in Table II and Figure 5, we developed the following equation to determine the postmetamorphic

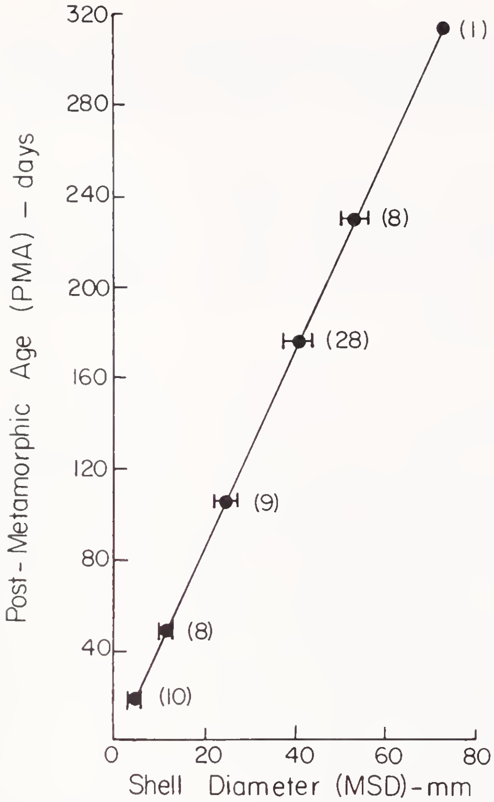

FIGURE 5. Determination of postmetamorphic age (PMA) based upon the equation given in text (see Discussion). The relationship in the equation is entirely independent of body weight. Numbers in parentheses are the number of determinations for each point plotted; standard error given for each shell size.

age (PMA) of *Aplysia*:

$$\text{PMA} = \frac{\text{MSD} - \text{SDMM}}{\text{RSG}};$$

where MSD is the maximum shell diameter (see Fig. 5); SDMM is the shell diameter at metamorphosis, 0.4 mm (see Kriegstein, 1977; Switzer-Dunlap and Hadfield, 1977); RSG is the rate of shell growth (See Table II). The PMA of the smallest animals described above is

$$\frac{4.6 \text{ mm} - 0.4 \text{ mm}}{0.23 \text{ mm/day}} = 18 \text{ days}$$

This age compares favorably with that of animals whose birthdate was known, 17 days after metamorphosis, as described by Kriegstein (1977, Table I).

It is now possible to determine the PMA of the age groups studied previously (Papka *et al.*, 1981; Rattan and Peretz, 1981; Peretz *et al.*, 1982), using the equation above or Figure 5. Young animals have an average shell diameter of 10.4 ± 3.5 mm ($n = 24$), with body weights from 2 to 20 g. Mature animals have an average shell diameter of 27.4 ± 4.4 mm ($n = 92$), with body weights from 100 to 250 g.

Old animals have a minimal shell diameter of 41.2 ± 3 mm (n = 21), with a body weight of 500 g. The average shell diameter for old animals was 47.6 ± 4 mm (n = 106), with body weights over 500 g. The PMA for the three age groups are: young, 43 days; mature, 117 days; minimal old, 177 days; and average old, 205 days.

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INTRASPECIFIC AGGRESSION AND POPULATION DISTRIBUTIONS OF THE SEA ANEMONE *METRIDIUM SENILE*

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ABSTRACT

Aggregations of the sea anemone *Metridium senile* in Monterey Harbor sometimes contained only one clone (genetically identical individuals) but often contained two or more intermingled clones. The frequent occurrence of mixed clonal aggregations was perplexing, because *M. senile* uses fighting ("catch") tentacles in intraspecific agonistic interactions. A photographic survey of 19 quadrats showed that mixed clonal aggregations of anemones persisted throughout the 3.5 year study. Locomotion by the anemones, low frequency of nonclonemate contact, low population density, and infrequent occurrence of fighting tentacles were eliminated as possible explanations for the persistence of mixed clonal aggregations. Laboratory studies revealed differences among clones in the frequencies of fighting tentacle inflation and of injury to nonclonemates. We believe that low expression of these aggressive traits might permit intermingling of clones. However, high expression of aggressive traits was not found consistently in clones which were not intermingled, and therefore may not cause the segregation of such clones. Apparently, the most important factor contributing to the intermingling of clones was habituation of anemones to nonclonemate contact. The decrease in fighting tentacle inflation observed during two, six-day laboratory experiments was not due to fatigue, because contact with unfamiliar nonclonemates renewed aggression. We believe that habituation to nonclonemate contact occurs *in situ*, and that loss of habituation must occur occasionally to account for the occurrence of fighting tentacles.

INTRODUCTION

Detailed studies of the agonistic behaviors of sea anemones and certain other anthozoans may clarify the importance of competition for space in benthic marine environments. Recent research shows that some anthozoans demonstrate remarkable agonistic behaviors, including interactions among neighboring species of corals (Lang, 1973; Wellington, 1980), interactions between corals and anemones (Sebens, 1976), and elaborate intraspecific battles among genetically different individuals in several sea anemone species. Some anemones use acrorhagi in aggression (Abel, 1954; Bonnin, 1964; Francis, 1973a, b, 1976; Brace *et al.*, 1975; Bigger, 1976, 1980; Williams, 1978; Brace and Pavey, 1978; Ottaway, 1978; Brace, 1981), and some use "catch tentacles" in aggression (Williams, 1975, 1980; Purcell, 1977a). Many of these anemone species reproduce asexually, forming groups of genetically

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identical individuals (clones). Aggression, when it occurs, is between individuals of different clones or of different species, and is probably important in competition for space.

In addition to having feeding tentacles, some individuals of the sea anemone *Metridium senile* have a few special tentacles that can be inflated to a length of 4 to 5 times the diameter of the tentacle crown. These tentacles, called "Fangtentakeln" originally by Carlgren (1929), and more recently "catch tentacles" (*e.g.,* Hand, 1955), were long believed to be used in feeding. However, since recent work has shown that these tentacles are not used in feeding (Purcell, 1977a), but are used in aggression (Williams, 1975, 1980; Purcell, 1977a), they are referred to in the present paper as fighting tentacles. New contact of the tips of feeding tentacles with any anemone causes fighting tentacle inflation, but nematocyst discharge and resulting injury occur only when a fighting tentacle tip contacts a genetically different anemone (Purcell, 1977a).

On wharf pilings in Monterey Harbor, *Metridium senile* clones sometimes are separated from neighboring clones by an anemone-free zone, forming "segregated" clones. Individuals along the borders of such an anemone-free zone have fighting tentacles, and their aggressive behavior is thought to maintain the segregated distribution of the clones (Purcell, 1977a). More frequently, however, aggregations of two or more clones occur where individuals intermingle without an anemone-free zone, forming "mixed" clones. Often many individuals in these mixed clone aggregations bear fighting tentacles. We expected that mixed aggregations would not persist because aggressive interactions are known to occur among nonclonemates. The present study was undertaken to determine whether these mixed aggregations persist, and if so, to examine behavioral characteristics of different anemone clones that could promote the aggregations of more than one clone.

MATERIALS AND METHODS

Observations of natural populations

The study area at Wharf No. 2, Monterey Bay, California, was in a well-circulated outer region of a port used for commercial fishing and pleasure boating. Haderlie and Donat (1978) have described the overall physical and biological environment at this wharf habitat. Anemone populations were on pilings that had been in place for about 50 years. In the present study, 19 perpetually submerged quadrat sites were selected with aggregations of *Metridium senile.* The sites measured 30 by 50 cm, and were chosen to represent a variety of clonal interactions among the anemones: three had anemones of only one identifiable clone, three had one clone with one nonclonemate anemone in its midst, four had roughly equal numbers of two or three mixed clones and few of these anemones had fighting tentacles, five had approximately equal numbers of two or three mixed clones and many of these anemones had fighting tentacles, and four sites had two segregated clones. Sites consisting of one predominant clone will be called "single clone"; sites with two or more clones intermingled will be called "mixed"; and sites with two clones separated from each other by an anemone-free zone will be called "segregated." Adjacent clones were distinguished by color, as described by Purcell (1977a), a characteristic which appears to be genetically determined, and is consistent within a clone (Hoffmann, 1976).

A numbered plastic tag nailed to the piling marked the bottom center of each site. Color transparencies were taken at each submerged site at six week intervals beginning in April, 1977. After five sampling periods, when such frequent sampling appeared superfluous, photographs were taken at twelve week intervals through April, 1978. Some of the quadrats were subsequently photographed at four to six month intervals through August, 1980.

Movements of anemones were monitored to test the extent to which the mixed clonal aggregations were labile. Individual anemones could often be identified between twelve week samples during the first year by their position relative to natural markers such as barnacles, and by their size, color, and possession of fighting tentacles. Small displacements were impossible to detect because orientation and expansion of the tentacle crowns varied. We used two hand-held slide viewers and a light table to optically superimpose and magnify two photographs taken of the same site on consecutive sampling dates.

Transparencies from the initial sampling were compared to those from a year later to determine changes in the percentage of anemones with fighting tentacles in each clone, and in the amount of nonclonemate contact. Transparencies were examined simultaneously as before, and at 7× magnification under a dissecting microscope to determine presence of fighting tentacles. Occasionally individual anemones were contracted such that the tentacles could not be seen, and therefore these individuals were not included in the analysis.

The percentage cover of anemones within an aggregation, including their tentacles, was used to assess the degree of crowding and tentacle contact. Percentage cover was quantified by viewing a color transparency of each study site with a dissecting microscope, and tabulating intercepts with anemones on a random array of points throughout the area of the slide (Kitting, 1980). Photographs taken of the sites after the first year of the study were examined using the same methods as before, but by then the original individual anemones could no longer be positively identified.

Laboratory observations of aggressive behavior

Anemones were collected from the pilings with a putty knife and kept isolated from other clones for 8 hours in flowing seawater. Undamaged anemones were then placed in plastic containers 10.5 cm square and 6 cm deep which had small holes near the top to permit water, but not anemones, to escape. Unfiltered sea water at 12–13°C flowed into a large plastic reservoir from which siphons supplied each container with approximately equal water flow. Anemones were fed larvae of brine shrimp, *Artemia salina*. The sediment and mucus accumulating in each container were vigorously rinsed away with sea water twice daily after each feeding. All anemones used in the experiments measured about 5 cm expanded column height.

Three sets of behavioral experiments were run. The first set used anemones from selected single and mixed clone quadrats at the end of one year of photographic sampling. These results were preliminary, and are used only to correlate field and laboratory results. The second and third sets of experiments, which did not use clones from monitored quadrats, tested clonal differences in aggression and tested habituation to nonclonemate contact. These anemones had five to eight fighting tentacles. Anemones were collected from five clones in mixed aggregations for both sets of experiments. Two anemones from each of two clones were placed in each container to form pairwise combinations of the different clones. Containers with clonemates provided information on aggressive activity without nonclonemate influence. Observations began 18 h after introduction of the anemones into the containers, when all anemones were attached and expanded. Anemones were observed during 0.5 to 1.5 h periods, totalling 11.5 h over 6 days, and 18 h over 5 days in the second and third sets of experiments, respectively. The clonemate and nonclonemate contacts of each anemone, the number of anemones with inflated fighting tentacles, and the number of fighting tentacles inflated per anemone were noted during each observation period. The injuries, visible as lumps of necrotic tissue, inflicted by fighting tentacles were also counted for each anemone daily.

If habituation to nonclonemate contact occurred, fighting tentacle inflations should decrease over time. However, such a decrease in aggression might also be explained by fatigue of the anemones due to experimental conditions. A test to determine whether the anemones were fatigued or habituated was conducted on the last day of both experiments. One hour of observations were made, then one anemone from a third clone (previously kept with clonemates in the laboratory) was placed in nonclonemate contact in each of several containers having pairs of other clones. Alternatively, an additional anemone from one of the two clones already present was introduced into each of several containers. Other containers remained unchanged for comparison. After the additional anemones had expanded, fighting tentacle inflations were monitored in all containers for 1 and 4 h in the second and third experiments, respectively.

Arcsine transformations used in calculations on percentages and statistical tests are from Sokal and Rohlf (1969).

RESULTS

Observations on natural populations

Mixtures of two or more clones of *Metridium* were not expected to persist unchanged, because laboratory observations of their aggressive interactions showed that the anemones continually injure, and can kill, nonclonemates over periods of a few weeks (Purcell, 1977a). Seven hypotheses were formulated which could explain the occurrence of mixed clonal aggregations.

Hypothesis 1: *Anemones may change location such that nonclonemates avoid prolonged interaction.* Analysis of periodic photographs at 12-week intervals tested for such locomotion. During the first year, the mean percentage of anemones that changed locations noticeably was very low, 5.8% at all sites over the entire year. Movement in mixed sites tended to be slightly higher than in the other sites (Table I), but the difference was not significant (single classification analysis of variance (ANOVA)). A few anemones in segregated sites crossed the anemone-free zone and then remained in nonclonemate contact. In one segregated site, many individuals of one clone traversed the zone between clones, eliminating the anemone-free space. Anemones at all other experimental sites changed location very rarely. Hypothesis 1 was rejected as an adequate explanation for intermingled clones.

Hypothesis 2: *Anemones in mixed aggregations may not remain in tentacle contact with nonclonemates.* Tentacle tip contact has been documented as a stimulus for aggression in *M. senile* (Purcell, 1977a). The number of anemones in contact with nonclonemates was compared between the beginning of the study and after one year for every clone in mixed, single clone, and segregated sites in a two-way ANOVA. The numbers of nonclonemate contacts in the three types of sites were significantly different (Table I). As expected, mixed sites had much greater nonclonemate contact ($P < 0.001$). However, no significant change in the overall number of nonclonemate contacts occurred between two samples taken one year apart ($P > 0.10$). The percentage of anemones in mixed sites in nonclonemate contact was 92.9% at the beginning of the study, and was 96.5% at the end. Hypothesis 2 was rejected.

TABLE I

Characteristics of anemone populations at quadrats monitored photographically for one year.

	Type of anemone aggregation			Statistical probability
	Single clone	Mixed	Segregated	
Mean % of anemones changing location during one year	3.9 ± 4.8	9.6 ± 1.9	4.0 ± 1.4	$P > 0.25$ NS
Mean % of anemones in nonclonemate contact	0	92.9 ± 10.0	9.2 ± 3.5	$P < 0.001$
Mean % of anemones with fighting tentacles	5.5 ± 15.0	21.5 ± 16.6	57.8 ± 5.2	$P < 0.001$
Mean % cover of anemones	96.3 ± 0.8	93.7 ± 2.2	98.2 ± 1.9	$P > 0.10$ NS
Number of anemones	154	213	231	
Number of clones	6	25	8	

Except for the percentage of anemones changing location, percentages are based on measurements at the beginning of the study. See text for comparisons over the year. The values are means ± standard deviations. The probabilities expressed are from tests (ANOVA) using the actual numbers (not percentages) for each quadrat. The null hypothesis in each case was that no difference existed among the three types of sites.

Hypothesis 3: *Percentage cover of the anemones in mixed aggregations might be low, and thereby might minimize feeding tentacle contact, keeping aggression infrequent.* The mean percentage cover for all aggregations was very high, averaging 96.1%, with mixed sites having only slightly lower cover (Table I). A comparison of the percentage cover of anemones within aggregations of single clone, mixed, and segregated sites showed that the sites did not differ significantly (ANOVA, $P > 0.10$). Low percentage cover cannot explain persistence of the mixed clonal aggregations. Hypothesis 3 was rejected.

Hypothesis 4: *Anemones from clones in mixed aggregations may rarely form fighting tentacles.* The percentage of anemones with fighting tentacles was greatest in segregated sites (Table I). This is due to the quadrat placement which focused on the opposing borders of two clones where the fighting tentacles are found (Purcell, 1977a). For one clone, mixed, and segregated sites, the number of anemones with fighting tentacles was compared at the beginning of the study and after one year (two-way ANOVA). The number of anemones with fighting tentacles differed significantly among the three types of sites ($P < 0.001$). However, between the beginning and the end of the first year, no significant overall change occurred in the number of anemones with fighting tentacles ($P > 0.10$). The percentage of anemones with fighting tentacles increased over the year from 3.5 to 11.8% at single clone sites, from 21.5 to 23.8% at mixed sites, and from 57.8 to 58.9% at segregated sites. Hypothesis 4 was rejected.

The preceding measurements of *M. senile* populations at experimental sites all indicate that both mixed and unmixed populations changed surprisingly little over one year, and individual anemones often remained at the same attachment site and maintained nonclonemate contact for at least a year.

Eleven of the original quadrats with mixed clonal aggregations were photographed at intervals throughout 3.5 years. In all cases, quadrats contained the same anemone clones at the end of the study as at the beginning. Locomotion by the anemones, while undetectable over short time periods, produced noticeable changes in the arrangement of anemones over 3.5 years (Fig. 1). However, no segregation

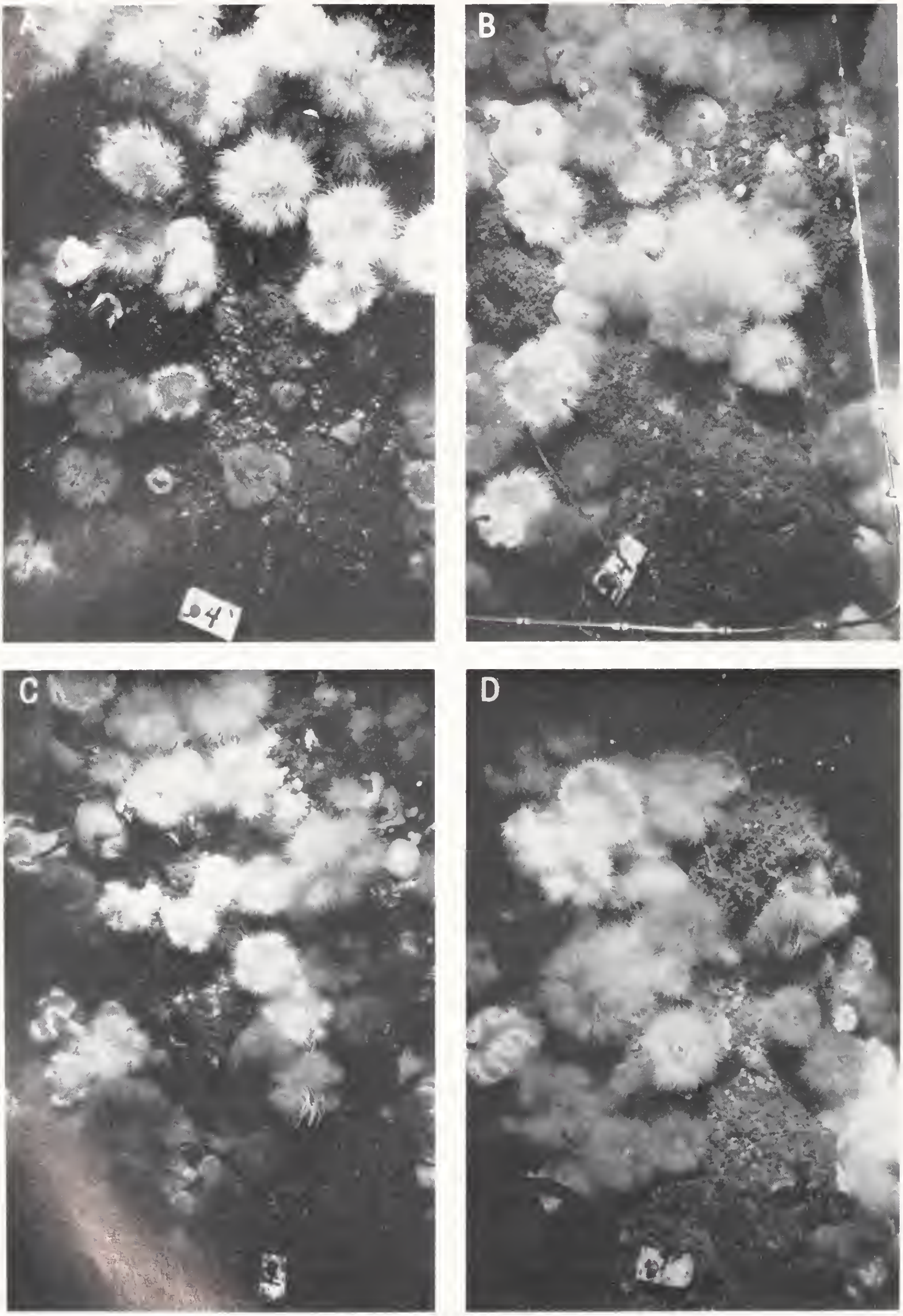

FIGURE 1. Photographs of one quadrat in a) May, 1977, b) April, 1978, c) January, 1980, and d) August, 1980. Anemones of three clones are intermingled; overall light gray, light gray with white banded tentacles, and orange (darker gray in the photograph) with banded tentacles. Fighting tentacles are the large, opaque tentacles near the mouths of some anemones. The scale markers in b) are 5 cm apart.

of the mixed clones occurred. Asexual reproduction and growth of the anemones, although not measured, undoubtedly contributed to the long-term changes in the photographed quadrats. These long-term observations upheld the conclusion from the detailed analysis over the first year, that mixed clonal aggregations persist with little change over time.

Laboratory studies of aggressive behavior

Hypothesis 5: *Clones may differ in the frequency of fighting tentacle inflation.* The numbers of inflated fighting tentacles were monitored in the laboratory, and related to population differences seen in clones from the photographed sites. In the field, the percentage of anemones with fighting tentacles in clones from mixed sites ranged from 0–100% (mean 21.5 ± s.d. 16.6%). In the laboratory, differences in behavior were obvious among the various clones; some clones had fighting tentacles inflated more frequently than did other clones (Fig. 2). However, anemones from clones having a high percentage of individuals with fighting tentacles did not necessarily inflate fighting tentacles frequently in the laboratory, nor was the reverse true (Fig. 2; $r = 0.32$, $P > 0.05$). One clone from a segregated site displayed one of the highest frequencies, even when in a container holding only clonemates. However, not all clones from segregated sites inflated fighting tentacles frequently, nor did all clones from mixed sites inflate them rarely (Fig. 2). This suggests that a higher frequency of fighting tentacle inflation in segregated clones was not sufficient to explain the anemone-free zone separating the two clones. Likewise, a low frequency of fighting tentacle inflation was not sufficient to explain the ability of clones to intermingle.

Anemones with fighting tentacles were taken from five different clones and used to test further for differences in aggressiveness among clones. The responses of each clone to the other clones paired with it in the laboratory, and the mean response

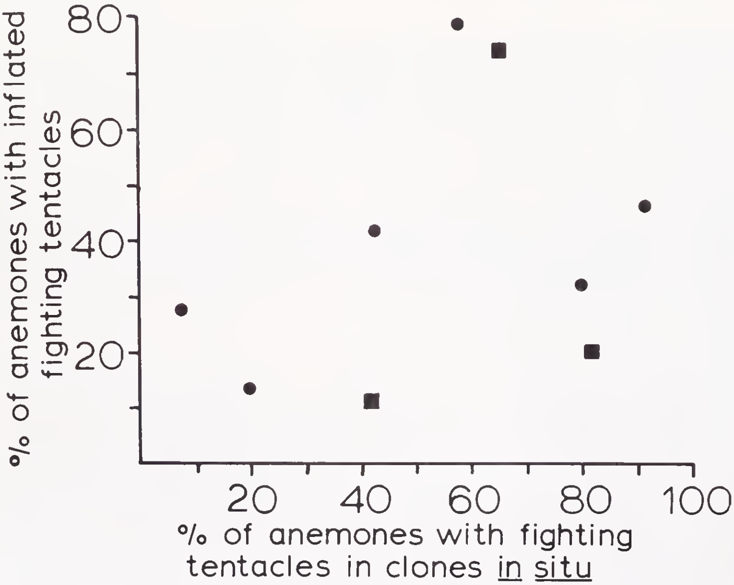

FIGURE 2. The percentage of anemones from different clones with fighting tentacles in their natural environment versus their inflation of fighting tentacles against nonclonemates in the laboratory. Squares = segregated clones, closed circles = mixed clones.

TABLE II

Comparison of fighting tentacle inflation among five anemone clones in experimental pairs.

		Anemones in "A" Clone with fighting tentacles inflated		
"A" Clone	"B" Clone	No.	Mean	No. of anemones in nonclonemate contact
1	2	11	9.50	13
	3	7		8
	4	14		18
	5	6		14
	1	1		
2	1	12	10.00	10
	5	8		11
	2	2		
3	1	6	3.75	7
	4	1		13
	5	4		16
	3	4		
4	1	4	2.33	18
	3	0		14
	5	3		15
5	1	5	5.50	15
	2	6		13
	3	9		17
	4	2		18

The numbers of anemones with fighting tentacles inflated were summed over 10.5 h of observation for each clone in each pair during the six day experiment.

for each clone, are given in Table II. A comparison of the number of these anemones with inflated fighting tentacles showed a significant difference among clones (ANOVA, $P < 0.025$). Clearly, the clones displayed different frequencies of fighting tentacle inflation. The responses of each clone might be expected to vary with the amount of nonclonemate contact experienced. However, the clones did not vary significantly in the number of anemones in nonclonemate contact during the experiment (ANOVA, $P > 0.25$). The differences among clones in the frequency of inflating fighting tentacles cannot be attributed to unequal nonclonemate contact. Hypothesis 5 was *not* rejected. Low frequencies of fighting tentacle inflation might contribute to intermingling of some clones.

Hypothesis 6: *Clones may differ in the infliction of injury upon nonclonemates.* Fighting tentacle adherence to a nonclonemate, with the resulting injury, causes the victim to inflate its fighting tentacles (Purcell, 1977a). This effect would be expected to escalate aggression in the experimental containers. The total number of fighting tentacles inflated over the six-day experiment were compared for each pair in the experimental clones (Fig. 3a). The number of fighting tentacles inflated was not significantly correlated between clones of a pair (Kendall Rank Correlation, $P > 0.05$). This is because the pairing of clones #1 and #4 differed substantially from the others. Both clones in each of the other pairs tended to show similar frequencies of fighting tentacle inflation, although (#1, #4) was an exception.

Injury might be a cause of enhanced aggression in nonclonemate interactions.

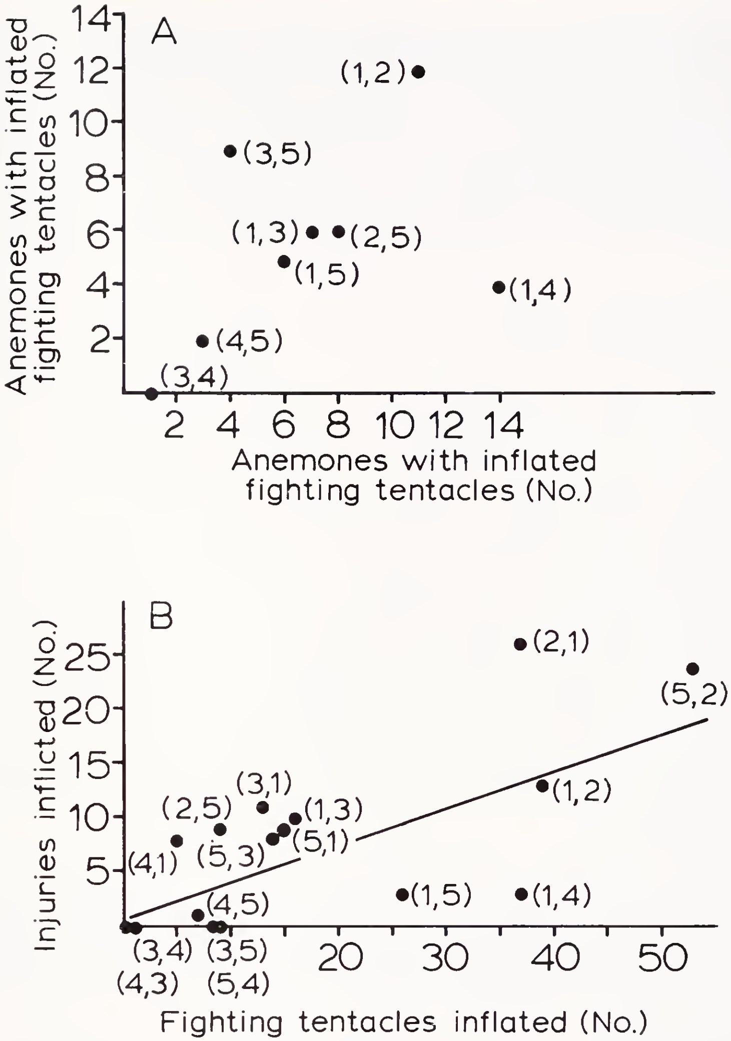

FIGURE 3. The effect of interaction between clones. Points are labeled with numbered clone pairs corresponding to the appropriate (x, y) coordinate numbers. Values represent totals for each clone during 11.5 h of observation. a) The number of anemones with fighting tentacles inflated tended to be similar for both clones in a pair with the exception of (1, 4). b) The number of fighting tentacles inflated by a clone was positively correlated with the injuries inflicted by that clone ($P < 0.005$).

The number of fighting tentacles inflated was significantly correlated with the number of injuries inflicted by each clone in each container (Fig. 3b, $P < 0.01$). Overall, more inflated fighting tentacles resulted in more injuries. However, in some clone pairs, fighting tentacle inflations were frequent, but injuries rare. In all clone pairs, fighting tentacles sometimes failed to adhere to nonclonemates even after prolonged contact of the fighting tentacle tip. Hypothesis 6 was *not* rejected. Infrequent injury to nonclonemates may contribute to the intermingling of some clones.

Hypothesis 7: *Anemones may habituate or adapt to nonclonemate contact.* The first set of behavioral observations showed a decline in fighting tentacle inflation over the course of the six day experiment. We use the term habituation to describe the observed decrease in response (fighting tentacle inflation) to a stimulus (nonclonemate contact). We have to assume that the quality of the stimulus is constant throughout the experiment. To test for habituation to nonclonemate contact, the number of anemones with inflated fighting tentacles per hour of observation was plotted for each day in two further sets of experiments (Fig. 4a). In one experiment, the number of anemones with inflated fighting tentacles per hour decreased from

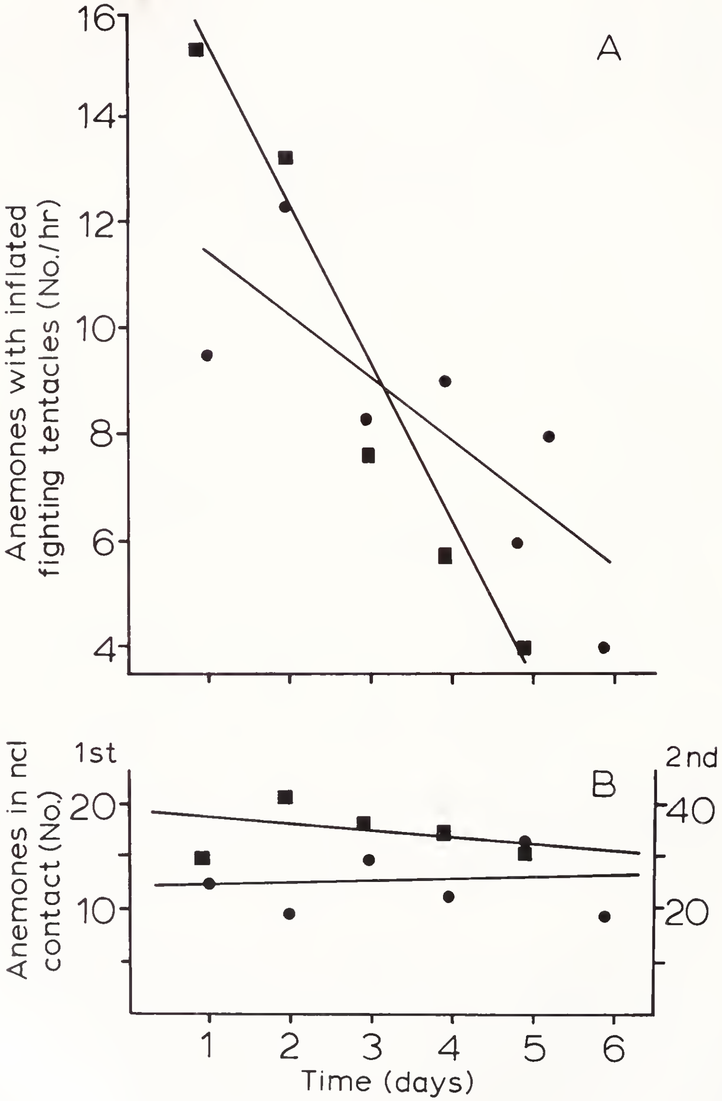

FIGURE 4. a) Decrease in aggression among nonclonemates confined together. Correlations for the two experiments: (●) $r = -0.79$, $P < 0.05$; (■) $r = -0.96$, $P < 0.01$. Each point equals the mean of 48 anemones. b) Frequency of anemones in nonclonemate contact during the same experiments. No significant change occurred over time.

peak activity of 12.3 anemones on the second day to 4.0 anemones on the sixth day. Similarly, the number of fighting tentacles inflated per hour decreased from 36 to 12. This strongly suggests a real decrease in aggression, although the decrease in the numbers of anemones fighting was not quite statistically significant (least squares regression, $P = 0.07$). The decrease in the number of anemones fighting in the next experiment was statistically significant (Fig. 4a, least squares regression, $P < 0.05$). At the same time, there was no significant decrease in the number of anemones in nonclonemate contact in either experiment (Fig. 4b), which eliminates reduced contacts as a cause of the reduced aggression.

To distinguish between fatigue and habituation as possible causes of the observed decrease in aggression, an anemone from a third clone was added to the established pairs of clones. If the observed decreases in aggression were due to fatigue, these new contacts should have resulted in continued low fighting tentacle activity. Instead, the mean number of inflated fighting tentacles increased dramatically in the containers which received a different nonclonemate (Table III). Within a few hours after contact with a new nonclonemate, much fighting tentacle activity occurred in anemones that had not inflated fighting tentacles for two or three days prior to the new contact. In containers to which no new anemones were added, the mean number of inflated fighting tentacles continued to decrease (Table III). Neither fatigue nor decreased nonclonemate contact accounted for the decreased fighting tentacle activity. These results indicate that the anemones habituated to nonclonemate contact. The addition of another anemone from one of the clones of an experimental pair caused some increase in aggression, but the sample size was too small to determine whether this was a significant effect (G-test, $P > 0.05$). It was not possible to determine from these results whether habituation was specific to the clones contacted. Hypothesis 7 was *not* rejected. Habituation appears to be a major factor contributing to intermingling of anemone clones.

DISCUSSION

Several characteristics of the sea anemone *Metridium senile* were investigated in an attempt to explain: 1) how clones can be intermingled in spite of aggression which is known to occur between nonclonemates having fighting tentacles, and 2) how anemones of some clones are intermingled, while other clones are segregated.

TABLE III

Experiment showing that habituation, and not fatigue can explain the decrease in aggression observed over six days in two experiments.

	Fighting tentacles inflated (No./h)		
	Before	After	G-test
A No anemone added (16)	18	13	A *vs* B NS
B Anemone of same clone added (5)	4	8	B *vs* C NS
C Anemone of different clone added (8)	7	66	A *vs* C $P < 0.005$

Aggression was measured before and after additional anemones were placed in selected containers. If fatigue had occurred, aggression would not be expected to increase upon addition of any nonclonemate. Fighting tentacle inflations by the added anemones are not included. The numbers of replicate containers, each with four anemones, appear in parentheses.

Dense aggregations of two or more clones were stable over 3.5 years of observation; nonclonemates with fighting tentacles remained in contact at percentage cover equal to the cover within single clone aggregations. Low frequency of nonclonemate contact due to locomotion by the anemones, avoidance of contact, low percentage cover, and lack of fighting tentacles were eliminated as possible explanations for mixed aggregations of anemone clones.

Locomotion in natural populations of solitary actinians has been studied by Dunn (1977) in *Epiactis prolifera* and by Ottaway (1978) in *Actinia tenebrosa.* Ottaway (1978) states that following intraspecific aggression, the wounded anemone moved directly away from the site of wounding. We are aware of no previously published long-term population studies of aggregating actinians.

Clones were found to differ in several characteristics which would affect the intensity of nonclonemate aggression. Clones differed in the proportions of anemones with fighting tentacles and in the number of fighting tentacles per anemone. In the laboratory, clones differed in the frequencies of fighting tentacle inflation and in infliction of injury. These differences were not due to the amount of nonclonemate contact. Differences in aggression previously have been shown among clones of the anemone *Anthopleura krebsi* (Bigger, 1980), and among color morphs of *Actinia equina* (Brace *et al.*, 1975) and *Phymactis clematis* (Brace, 1981). A low expression of any of these characteristics could result in low levels of aggression in particular nonclonemate interactions. The intensity of aggression would depend upon the interaction of the clones involved.

Anemones from segregated clones did not display consistently high levels of the tested aggression traits that might make them particularly incompatible with other clones. These anemones seemed to habituate to nonclonemate contact, although the possibility that some clones might habituate less readily than other clones was not tested. It seems likely that segregated clones are a result of established, dense clones growing until they confront each other along a border, where nonclonemate interactions then cause fighting tentacles to develop (Purcell, 1977a) and an anemone-free zone to form. Mixing was observed between two previously segregated clones, hence segregation may be only temporary.

Aggression decreased between experimental pairs of *M. senile* clones kept together for 6 days in the laboratory. This may be the most critical factor in promoting mixed clonal aggregations of anemones. We use the term habituation to describe the observed decrease in fighting tentacle inflation. We do not intend to imply an understanding of the mechanisms involved, which conceivably could involve loss of the tendency to aggress (habituation, sensory adaptation), or loss of the stimulus to aggress (anemones becoming unrecognizable as nonclonemates). Habituation to mechanical stimuli has been experimentally established in *Hydra* (Rushforth, *et al.*, 1963) and in the sea anemones *Aiptasia* (Jennings, 1905) and *Metridium* (Allabach, 1905). Bonnin (1964) and Bigger (1980) found that the threshold for induction of the acroraghial response initially decreased, but then increased after repeated induction. These two examples suggest habituation, but fatigue was not eliminated as a possible explanation. Bigger (1980) cautions that not all specimens became totally refractory, and that thresholds remained low at induction intervals of 15 min or more. For *M. senile*, we show that contact by a nonclonemate elicited aggression in anemones habituated to another nonclonemate clone, thus showing that the anemones were not fatigued.

The persistence of mixed clonal aggregations of anemones and the fact that anemones habituate to nonclonemate contact are contrary to expectation, if intraspecific aggression is important in competition for space. Several intriguing questions are raised: (1) How important is intraspecific aggression in competition for space? (2) Since nonclonemates habituate, how are fighting tentacles maintained in the populations? (3) What advantage, if any, is conferred to the anemones by aggregating, even with nonclonemates?

In this 3.5 year study, there was very little apparent effect of intraspecific aggression on populations of *M. senile*. The Monterey wharf pilings appear to support far greater population densities of *M. senile* than do natural substrata (pers. obser.), and presumably would be subject to greater competition for space. However, fighting may be advantageous only if there is free space available. Intermixed nonclonemates in dense aggregations could continually battle each other with little to gain, unless one clone was able to kill off the other clones. Death as a result of fighting tentacle battles is not common in the laboratory, and apparently rare in nature. The importance of these battles may be greatest in causing the retreat of nonclonemates in areas where free space is available. Therefore nonclonemate habituation would ameliorate useless aggression where space is unavailable.

We believe that loss of habituation or adaptation to nonclonemate contact must occur in nature, because the anemones do have fighting tentacles and aggression does occur. *Metridium senile* exhibits periodic phases of contraction and extension which are related to light, feeding, and tidal cycles (Batham and Pantin, 1950; Robbins and Shick, 1980). Anemones in the field might contract periodically and therefore break contact with nonclonemates long enough to cause loss of habituation.

Francis (1973a) proposed several advantages of compact clonal organization for the intertidal anemone *Anthopleura elegantissima*. Some of these explanations could be modified to suggest advantages that might apply to aggregations of *M. senile*. Perhaps these anemones benefit more by aggregating, even with nonclonemates, than by keeping clones separated by aggressive interactions. The following possible benefits include: (1) minimizing or utilizing water motion—body size, shape, and behavior affect water flow patterns and drag forces exerted on benthic cnidaria (Wainwright and Koehl, 1976), and aggregating may reduce shearing forces of the water on individual anemones; (2) cooperative feeding—adjacent individuals of *M. senile* have been observed participating in the capture of food larger than single anemones could consume (Purcell, 1977b); (3) preventing larval settlement by potential space competitors—this could be effected by consumption of the larvae and by actual occupation of the available substratum; (4) reducing predation—the nudibranch *Aeolidia papillosa* is an important predator upon *Metridium* (Stenouwer, 1951; Waters, 1973; Harris, 1976). Aggregation of anemones may serve to reduce such predation by making a large part of the population less accessible to predators. Similar advantages for "colonial" growth of marine invertebrates were advanced by Jackson (1977), and these apply as well to aggregating anemones.

The arrangement of segregated clones of *Metridium senile* is like the clonal arrangement of *Anthopleura elegantissima*. Unlike *M. senile* clones, intermingling of *A. elegantissima* clones does not seem to occur (L. Francis, 1973a, and pers. comm.). Possible factors contributing to this difference in clonal arrangements of these two anemone species include the following: (1) *A. elegantissima* appears to remain in close clonemate contact after asexual reproduction (Francis, 1973a); and, (2) *A. elegantissima* may not habituate to nonclonemate contact (N. Withers, pers.

comm.). Because *A. elegantissima* clones remain discrete while *M. senile* clones often do not, it is tempting to speculate that evolutionarily, intraspecific competition for space may have been more important in *A. elegantissima* habitats.

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HORMONAL CONTROL OF REPRODUCTION IN *BUSYCON*: II. LAYING OF EGG-CONTAINING CAPSULES CAUSED BY NERVOUS SYSTEM EXTRACTS AND FURTHER CHARACTERIZATION OF THE SUBSTANCE CAUSING EGG CAPSULE LAYING

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ABSTRACT

Ram (1977) previously observed that *Busycon* would lay empty egg capsules when injected with nervous system extracts and suggested that egg capsules containing eggs would be laid only after a number of eggless capsules had first been laid. Data supporting this suggestion and characterizing the gel filtration behavior and species specificity of the substance causing capsule laying are reported.

Busycon capsule strings collected in the field always had empty egg capsules at the initially laid end (*B. carica*, 13–17; *B. canaliculatum*, 4–57). *B. carica*, collected while laying in the field, continued to lay capsules in the laboratory at the average interval of 1.9 ± 1.5 hours/capsule.

Injection of nervous system extracts into *B. canaliculatum* caused capsule laying. The least amount that would cause capsule laying was $^1/_{16}$ of a nervous system. Injection of ¼ nervous system every two or three hours resulted in the laying of egg-containing capsules after a series of four or more empty capsules. The number of initial empty capsules was correlated with the percent of injections causing capsule laying. The percentage of hard capsules increased from 36 ± 24% before eggs were inserted to 59 ± 27% after eggs were inserted ($P < 0.05$).

The substance causing capsule laying in *Busycon* eluted from Sephadex G-50 at the same position as *Aplysia* egg laying hormone; however, cross-injection experiments between the two species failed to cause egg or capsule laying. Nervous system extracts from *Strombus gigas* caused capsule laying in *Busycon*; whereas, *Busycon* nervous system extracts did not cause laying in *Strombus*.

INTRODUCTION

Large prosobranch gastropods, including *Busycon, Strombus,* and *Haliotis*, are commercially important sources of protein and ornamental shells. Efficient exploitation of these animals, and possibly development of mariculture for them, may depend on increased knowledge of mechanisms controlling their reproduction, the knowledge of which might be exploited to produce spawn at desired intervals, in increased numbers, and/or beyond the natural spawning seasons. For *Haliotis*, spawning can be reliably triggered by exposure of ripe females to hydrogen peroxide in alkaline sea water (Morse, *et al.*, 1977), a method that did not work in *Strombus* (Morse, *et al.*, 1978). For *Busycon*, Ram (1977) reported that injection of nervous

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Abbreviation: central nervous system without the visceral ganglion, dissected along with a short piece of esophagus, CNS.

system extracts caused the laying of egg capsules, which were, however, devoid of eggs. In the present report, a method is demonstrated for obtaining egg-containing capsules in *Busycon.* In addition, the substance that induces the laying of egg capsules by *Busycon* is further characterized, and the species specificity of nervous systems inducing the response is examined.

In the field, *Busycon* ordinarily lays long strings of egg capsules over periods of several days (Magalhaes, 1948). This contrasts with the laying of egg capsules caused by nervous system extracts, in which usually only a single eggless capsule is laid in response to the injection of an extract of one quarter of a nervous system. Ram (1977) proposed that insertion of eggs into egg capsules might occur only when the substance(s) causing egg capsule laying had been working over longer periods than were used in his experiments. According to this proposal, an animal would be expected to lay a number of eggless capsules (perhaps 5 to 15), on the first day of capsule laying, before eggs would be inserted in subsequent capsules. Two capsule strings laid spontaneously in the laboratory seemed to confirm this proposal, since the initial 8–11 capsules laid during the first day of laying contained no eggs, after which eggs appeared in increasing numbers in subsequent capsules.

Although empty capsules in *Busycon* egg strings had been noted by Magalhaes (1948), she gave no indication of the location of the empty capsules in the string. The location of the empty capsules is critical to this proposal; there must be several at the beginning of the string. Therefore, the first data presented concerning this proposal are on the distribution of the number of eggs in egg capsule strings collected in the field. A more direct test of the proposal is to inject animals with active nervous system extracts a greater number of times per day than in previous experiments to see if eventually, after laying a number of empty capsules, the animals would begin laying capsules with eggs. In the experiments of Ram (1977) the maximum frequency and number of injections in a day was two injections spaced three hours apart, and no eggs were obtained. When the number of injections per day was increased to eight to twelve, as described in this paper, capsules containing eggs were laid.

MATERIALS AND METHODS

Capsule strings of both *Busycon canaliculatum* and *Busycon carica* were collected in Nantucket Sound in July, 1978 and August, 1981. The collection area was near Cotuit Bay in a relatively homogeneous, current-swept sandy region in depths ranging from 1–4 meters. This area is about 22 km east of Woods Hole on the south side of Cape Cod, Massachusetts.

The end of the capsule string attached to the substrate was noted and the number of eggs per capsule was counted at regular intervals along the string. Observations in the field of animals still in the process of laying capsule strings clearly showed that the end of the capsule string attached to the substrate was laid first. Several *B. carica* still in the process of laying eggs were brought back to the laboratory to observe the capsule-laying rate.

Experiments were done at the Marine Biological Laboratory, Woods Hole, Massachusetts during July and August, 1981. Specimens of *Busycon canaliculatum*, collected locally by the Marine Biological Laboratory Marine Resources Department, were maintained in running sea water at the ambient temperature of 20–25°C.

Nervous systems for extracts were obtained from animals having a shell length greater than 13 cm, without regard to sex, since previous studies (Ram, 1977) had shown that nervous system extracts from both sexes could induce capsule laying. For all experiments, extracts were made from the central nervous system without the visceral ganglion, dissected along with a short piece of esophagus as described previously (Ram, 1977), and designated CNS. Dissected CNSs were immediately placed on ice and frozen at −15°C until used in making an extract. Eight to twenty-four CNSs were thawed, homogenized in a motor-driven glass-teflon homogenizer on ice with 0.1 to 0.3 ml filtered sea water per CNS, and then placed on a boiling water bath for 10 min. After cooling, the boiled homogenate was centrifuged 25 min at 10,000 × *g*, the supernatant was diluted with sea water to obtain 0.3 or 0.4 ml/one quarter CNS, and then this CNS solution was pipetted into several test tubes (usually 3 or 4 times the volume of one quarter CNS in each test tube), which were then stored at −15°C until needed for injection into recipient animals. Animals were injected through the side of foot, as described previously (Ram, 1977).

Since large *Busycon* are always female (Ram, 1977), large animals (shell length greater than 17 cm, wet weight without shell = 150–300 g) were used as recipients of nervous system extract injections. Initially, thirty large females were screened to find animals that would lay in response to injection of one quarter CNS. Not all large females lay in response to nervous system extract injection, a finding that is related in some animals to lack of sexual maturity (small gonad index, Ram, 1977); however, animals that respond once are usually responsive to subsequent injections (Ram, 1977). Thus, this screening procedure identified responsive animals to be used as recipients in further experiments.

For gel filtration of nervous system extracts, eight CNSs were homogenized and boiled in 1 ml sea water, as above. The aqueous phase after boiling was centrifuged at 20,000 × *g* for 25 min. Gel filtration of 200 μl of the supernatant was done on Sephadex G-50 equilibrated in sea water in a 0.8 × 29 cm polystyrene column. Fractions were approximately 1.2 ml (one run) and 0.8 ml (a second run). Optical density of all fractions at 280 nm was measured, and all fractions were frozen at −15°C until later bioassay. To bioassay fractions for ability to cause capsule laying the entire fraction was injected into recipients. Elution pattern was compared to the elution of marker proteins, *Aplysia* egg-laying hormone, and blue dextran run on a Sephadex G-50 column of identical dimensions (Ram, 1982a).

The species specificity of the substance causing capsule laying was examined by injecting nervous system extracts of *Aplysia californica* and *Strombus gigas* into *Busycon*, and *vice versa*. *Aplysia californica* (300–500 g) was obtained from Pacific Biomarine Laboratories. The abdominal ganglion of *Aplysia* is known to contain a 4385 dalton polypeptide that causes egg laying upon injection into mature *Aplysia* (Chiu *et al.*, 1979). Sea water homogenates of abdominal ganglia (one ganglion per ml) from *Aplysia* were made, and seven *Busycon* were injected with 0.5 ml each. As described in results, 0.5 ml aliquots of similarly prepared homogenates injected into *Aplysia* showed that sufficient egg-laying hormone to cause egg laying in *Aplysia* was present in such homogenates. Conversely, 0.5 ml aliquots of *Busycon* extracts containing one CNS per 0.5 ml were injected into each of four mature *Aplysia* which were known not to have laid eggs for at least 48 hours before injection. For these experiments *Aplysia* were maintained for three days in running sea water at ambient temperatures and were fed lettuce.

Strombus were obtained from C. Berg (Marine Biological Laboratory) who collected them in the Bahamas in August, 1981. *Strombus* CNS extracts, containing one CNS per 0.5 ml, were prepared in a manner identical to that described for *Busycon* extracts, and 0.5 ml was injected into each of three *Busycon*. Conversely, 0.5 ml of *Busycon* extracts containing in one experiment ¼ CNS per 0.5

ml and in another experiment one CNS per 0.5 ml were injected into each of six *Strombus.*

RESULTS

Spontaneously laid capsules: location of eggs in capsule strings and rate of laying

Capsules at the beginning of egg strings were always devoid of eggs. The distribution of egg number over several entire capsule strings in *B. carica* (n = 5 strings) and *B. canaliculatum* (n = 3) is shown in Figure 1. The median number of empty egg capsules at the beginning of a string was 15 (range 13–17) for *B. carica* and 5 (range 4–57) for *B. canaliculatum.* Additional features of the pattern are (a) the number of eggs reaches a plateau (*i.e.* egg numbers neither increasing nor decreasing more than 5–15 eggs/capsule) by about capsule number 20 or 30, and (b) at the end of the string the number of eggs/capsule either gradually

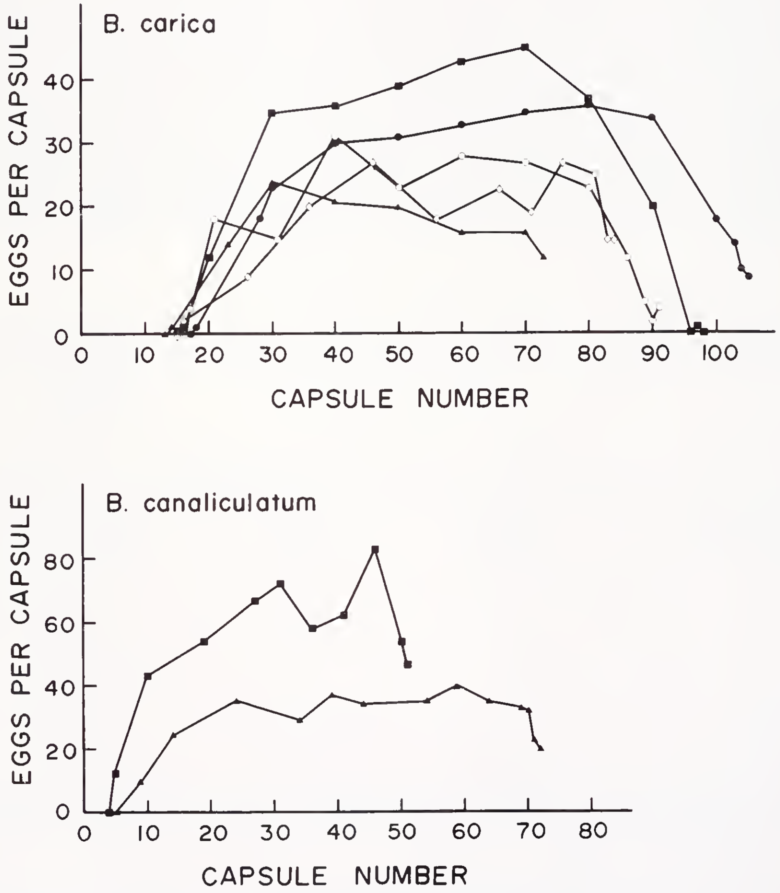

FIGURE 1. Distribution of eggs in *B. carica* and *B. canaliculatum* capsule strings collected in the field. The first point in each curve is for the last capsule containing no eggs; all previous capsules had no eggs. For *B. canaliculatum*, one other capsule string, containing 57 capsules, had no eggs in any capsule.

decreases to near zero over the last 10 to 20 capsules, or alternatively, capsule laying ends abruptly at or near the plateau level of eggs.

Capsule laying rates were observed in six *Busycon carica* brought back to the laboratory. The average interval between laying egg capsules was 1.9 ± 1.5 hours (Table I).

Screening recipients and dose-response analysis

As in the study by Ram (1977), not all large females laid egg capsules in response to injection of nervous system extracts. In the present study, injection of one quarter CNS caused capsule laying in response to the first injection in 9 of 30 recipients (30%). Animals that had been induced to lay once usually laid in response to subsequent nervous system extract injections, although as described below, some were more reliable than others. Experiments described below used only animals chosen from these nine "reliable layers."

To test the possibility of using a dose lower than one quarter CNS, which was the usual amount used by Ram (1977), dose-response analysis was done. CNS extracts prepared by the standard procedure were serially diluted in sea water to produce extracts containing $^1/_{64}$, $^1/_{32}$, $^1/_{16}$, $^1/_8$, and $^1/_4$ CNS per 0.5 ml. Six recipients were each injected with 0.5 ml of these solutions, starting with the lowest concentration and increasing the concentration on each subsequent injection, with injections made no more frequently than once every four hours. The number of recipients laying at each dose was as follows: 0, $^1/_{64}$; 0, $^1/_{32}$; 2, $^1/_{16}$; 4, $^1/_8$; 6, $^1/_4$. Thus, the threshold dose to induce capsule laying was between $^1/_{16}$ and $^1/_4$ CNS. Consequently, it seemed inadvisable to inject less than $^1/_4$ CNS in subsequent experiments as lower concentrations would be at or near the point at which capsule laying would fail.

Laying of egg-containing capsules caused by injections

Busycon were injected repeatedly with one quarter CNS every three hours (Experiment I) or every two hours (Experiment II). These rates of injection were

TABLE I

Average interval between laying capsules during spontaneous capsule laying.

Animal number	Number of capsules	Time to lay (h)	Hours/capsule
1	10	16	1.6
2	7	10	1.4
3	39	88	2.3
4	11	4	0.4
5	6	6.5	1.1
6*	34	156	4.6
Mean ± S.D.	18 ± 15	47 ± 62	1.9 ± 1.5

* Number of capsules was also counted for this animal 67 hours after bringing the animal into the laboratory, at which time 19 additional capsules had been laid, *i.e.* an average interval of 3.5 hours/capsule.

B. carica, collected while laying in the field, continued to lay in the laboratory. The number of capsules in the string was counted when animals were first brought into the laboratory, and the total number of capsules was counted again shortly after capsule laying was completed. The table gives the difference of these two numbers. This may have overestimated the number of capsules laid during the period of observation by one to three, since several capsules are normally obscured by the foot during laying.

chosen because they were comparable to the frequency of capsule laying observed in spontaneous capsule laying. As described below, the higher rate of injection was more successful at causing the insertion of eggs into egg capsules.

Experiment I. The four most sensitive recipients from the dose-response experiment were injected with one quarter CNS every three hours for 66 hours. Three of the four animals laid capsules after every injection; the fourth laid capsules after 17 of 22 injections (77%) and in no case missed laying two times in a row. Of the former animals, one began putting eggs in egg capsules at capsule number fourteen, which contained nine eggs. The number of eggs per capsule in subsequent capsules was 0, 13, 6, 7, 30, 0, 8, 9, and not counted. No other animal laid egg-containing capsules.

Experiment II. Six animals were recipients, including two animals from the previous experiment (one which had laid eggs and one which had not). Animals were injected with one quarter CNS every two hours for up to 42 hours. Injections into egg-laying animals were discontinued when three or more egg containing capsules had been laid. Since the latency to induce capsule laying by extract injection is greater than two hours (Ram, 1977), capsules did not appear until after the second injection (presumably the capsule caused by the first injection). After the last injection, two capsules were laid about two hours apart. Taking this delay into account, three animals, including the two from the previous experiment, laid capsules after every injection. Two other recipients laid capsules after 85%, 75%, and 69% of injections, and only the last animal missed more than one capsule in a row.

Five of the six recipients in this experiment began putting eggs in capsules after laying several empty capsules. The number of eggs per capsule for each egg layer is plotted in Figure 2. The median number of initial empty capsules was six (range 4–9). The number of eggs per capsule varied from one up to 82. The animal that

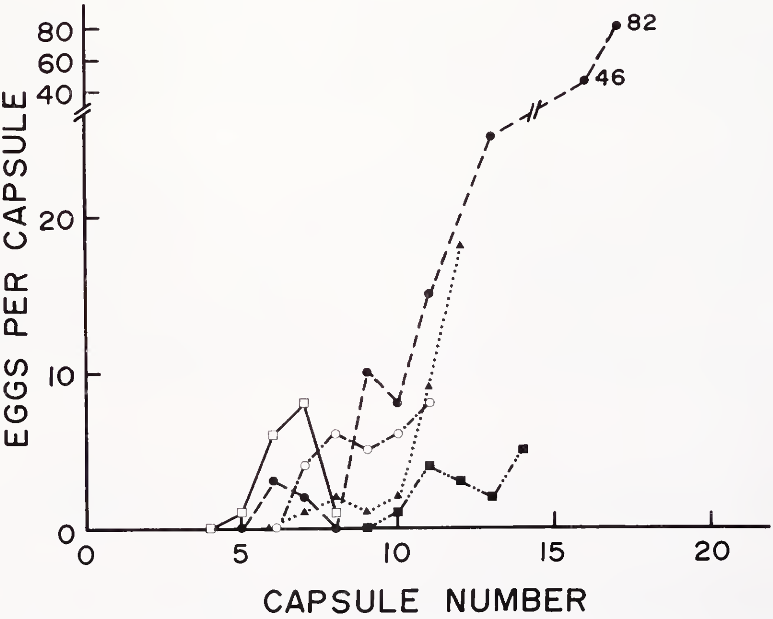

FIGURE 2. Number of eggs per capsule for capsules laid in response to injection of extracts of one quarter CNS every two hours. The first point in each curve is for the last capsule containing no eggs; all previous capsules had no eggs.

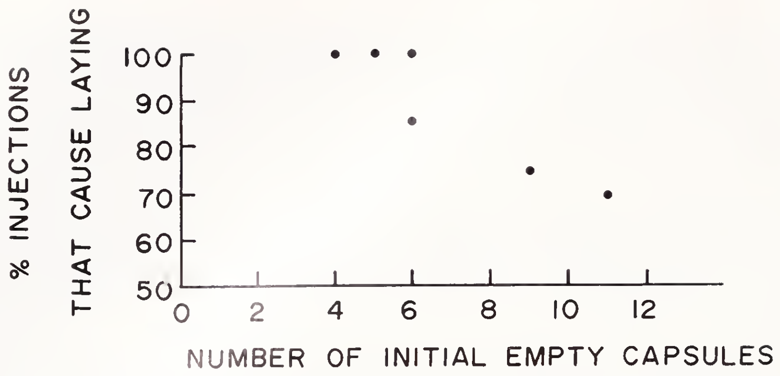

FIGURE 3. Correlation between the percent of injections that caused capsule laying and the number of empty capsules laid prior to laying capsules with eggs, for animals injected with one quarter CNS every two hours. The data illustrated include a point (69%, 11 capsules) for one animal which laid only eggless capsules (eleven) and for which only 16 injections were given. Conceivably, a larger number of empty capsules would have been laid if injections had been continued. Using the data illustrated, r = −0.93 and $P = 0.003$. Even if a larger number than 11 eggless capsules is used for the animal that did not lay eggs, the P level is <0.05 if the number of eggless capsules is <40. Without this point, the statistics for the correlation are r = −0.87, $P = 0.02$.

did not put eggs into its capsules was the one that laid capsules only 69% of the time; injections into this animal were discontinued after 16 injections. There is a significant correlation ($P < 0.05$) between probability of laying an egg capsule and the number of initial empty capsules (Fig. 3).

Shape of the capsules. As in the study of Ram (1977) animals laid either hard well-formed capsules or soft bulb-like capsules, depending on the success of the animal in passing the capsule into the pedal pore. Eggs were inserted into both hard and soft capsules; however, the proportion of capsules that were hard increased significantly after the animals began inserting eggs. Overall, the percentage of hard capsules increased from 36 ± 24% (mean ± standard deviation) before eggs were inserted to 59 ± 27% after eggs were inserted ($P < 0.05$, Wilcoxon signed ranks test).

The observation that egg-filled capsules were more likely to be hard probably cannot be explained simply by supposing that the animal becomes more likely to make hard capsules with each succeeding capsule. For example, for the animals that eventually inserted eggs in their capsules there was no significant difference in the percent of hard capsules among the first half of the initially laid eggless capsules (33 ± 27%) and the second half of the initially laid eggless capsules (38 ± 22%). Moreover, three animals that laid only eggless capsules did not lay more hard capsules at the end of the experiment than at the beginning.

Gel filtration

Figure 4 shows the optical density pattern of *Busycon* nervous system fractions eluted from a Sephadex G-50 column. Bioassays were done on all fractions eluting from just before the void volume peak to three fractions after the amino acid peak, and egg capsule laying was obtained only from fractions for which $V_e/V_0 \simeq 1.5$ (n = 2 column runs). The substance causing capsule laying eluted from the column at approximately the same place as would be expected for *Aplysia* egg-laying hormone.

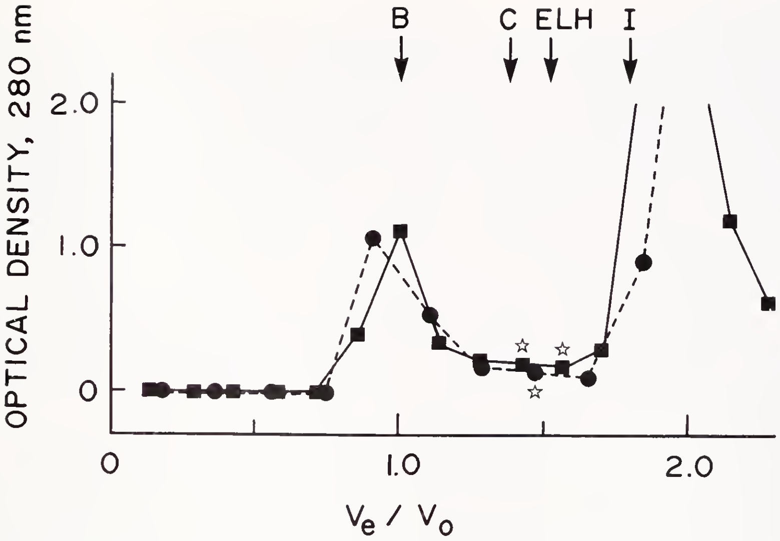

FIGURE 4. Sephadex G-50 gel filtration in sea water of *Busycon* nervous system extracts. The optical density of fractions from two separate runs (● and ■) is shown, and fractions that caused capsule laying upon injection into reliably laying *Busycon* are indicated (☆). For each run, all fractions from just before the void volume peak to three fractions after the amino acid peak were tested in the bioassay. The elution volume for markers run on a Sephadex G-50 column with identical dimensions and eluant (sea water) are indicated at the top: B, blue dextran (void volume); C, cytochrome c, 11,400 daltons; ELH, *Aplysia* egg-laying hormone, 4385 daltons; I, insulin B chain, 3,540 daltons.

Species specificity

Aplysia. Injection of one *Busycon* CNS into each of four *Aplysia* failed to cause egg laying. Three of the four recipients were tested three hours later with *Aplysia* abdominal ganglia extracts (one half ganglion per recipient), and all three began laying within 90 min. Thus, *Busycon* CNS extracts differ significantly from *Aplysia* abdominal ganglia extracts in causing egg laying in *Aplysia* (P = 0.028, Fisher exact probability).

Injection of half of an *Aplysia* abdominal ganglion into each of seven *Busycon* failed to cause capsule laying. All seven *Busycon* laid capsules on subsequent injection of *Busycon* CNS extract. Thus, *Aplysia* abdominal ganglia extracts differ significantly from *Busycon* CNS extracts in causing capsule laying in *Busycon* (P = 0.00029, Fisher exact probability).

Strombus. Injection of one *Strombus* CNS into each of three *Busycon* caused laying of empty egg capsules by two of the recipients. This result is thought to be highly significant since a false positive result has never occurred in any previous experiment; *i.e.* in all cases where responsive *Busycon* were injected with a solution known to contain little or no nervous system extract, no capsule laying was obtained (*e.g.* sea water and phosphate controls in Ram (1977); low doses in the dose-response analysis and pre-void volume fractions in gel filtration studies in this paper).

In the opposite direction, neither ¼ *Busycon* CNS nor one *Busycon* CNS injected into each of six recipient *Strombus* caused the laying of eggs or egg capsules. Failure to elicit laying in *Strombus* following *Busycon* CNS injection could have been due to insufficient dosage or to a lack of readiness to respond in the recipients.

DISCUSSION

The present study examined the proposal that *Busycon* would insert eggs into egg capsules only after a number of empty egg capsules were laid. The results support this proposal with both field and experimental data, showing (a) in the field, *Busycon* laid four or more empty capsules before egg-containing capsules were laid, and (b) when injected once every two hours with nervous system extracts that cause capsule laying, *Busycon* began laying egg-containing capsules after 4 to 9 empty capsules were laid.

A correlation was found between the number of initial empty capsules and the overall probability of laying egg capsules, and might be explained by supposing that the reproductive state of the animal in some way could influence both. Alternatively, this observation may have the trivial explanation that slow animals simply were not being injected as efficiently as fast ones, a circumstance that would be expected to decrease the number of capsules as well.

The correlation of the probability of hard capsules with the insertion of eggs is not so easily explained. Is there some internal feedback by which the animal senses when eggs have been inserted and therefore tries harder to move the capsule to the pedal pore? Whatever the explanation, it appears to be important for development of the eggs, as eggs in soft capsules degenerated, and such capsules fell apart within two weeks of being laid.

If the procedure developed here is to be of use in bringing *Busycon* reproduction under experimental control, it is necessary that the eggs be viable. Due to limited time, it was not possible to do a thorough study of this; however, it was observed that of the eggs laid in hard capsules in Experiment I, about half the eggs in each capsule had developed to the four cell stage by 10 days after laying. This rate of division is comparable to that seen in spontaneously laid eggs (Jonathan P. Davis, unpublished observations).

When capsules from Experiment II were maintained for 30 days, bacteria apparently invaded most of them; however, several contained normally developed young *Busycon.* This rate of development is comparable to that seen by one of us (Jonathan P. Davis, unpublished observations) in *B. carica* capsule strings freshly laid in the laboratory. Thus, it appears that eggs laid as a result of nervous system extract injections are fertile and capable of developing normally. Further experiments on optimal conditions for achieving this seem appropriate.

Induction of egg laying by injection of nervous system extracts has previously been demonstrated in *Aplysia* (Kupfermann, 1970; Toevs, 1970), *Pleurobranchaea* (Davis, *et al.*, 1974; Ram, *et al.*, 1977), *Lymnaea* (Geraerts and Bohlken, 1976), *Stylocheilus* (Ram, 1982a, b), and *Dolabrifera* (Ram, 1982b). The egg laying of these gastropods in response to nervous system extracts differs from the behavior of *Busycon* in two significant ways: (a) eggs are laid in response to a single injection, and (b) the egg-laying episodes induced by nervous system extracts are as complete as spontaneously occurring egg laying (*e.g.* in *Aplysia*, bag cell extracts induced the laying of egg masses as large or larger than spontaneously laid egg masses; Pinsker and Dudek, 1977). In contrast, *Busycon* requires several injections before eggs are laid, and the egg laying episodes end abruptly when injections are discontinued, as opposed to the several days long egg-laying episodes that occur spontaneously (Magalhaes, 1948).

The differences between *Busycon* and the other gastropods may reflect different patterns of neurosecretion. In *Aplysia* (Kupfermann and Kandel, 1970) and *Lymnaea* (Vlieger *et al.*, 1980) hormone is secreted from neurons during synchronized electrical activity of several minutes duration, after which the neurosecretory cells are quite inexcitable. Hormone is thus released in a comparatively short "bolus", comparable to that produced by an injection, and, therefore, the egg laying produced by neurosecretion and injection is comparable. In *Busycon*, the substance(s) causing egg laying apparently must be secreted over the entire episode, a situation differing from the short duration of action produced by injection of an extract.

This hypothesis raises interesting questions concerning the mechanism by which such secretion might be maintained over several days. Also, in view of the fact that the minimum effective dose to cause capsule laying was at least $^1/_{16}$ CNS, mechanisms that increase the synthesis of the substance causing egg laying may be necessary in order to have enough material to secrete over the entire episode. Alternatively, there may be more than enough material present for the entire egg laying episode, and the high dose (*i.e.* greater than $^1/_{16}$ CNS) needed in injection experiments may result from inactivation of the active agent during preparative procedures, lower efficiency of injection in the foot compared to the natural pattern and location of secretion, and/or lower sensitivity of the animal to the substance causing capsule laying than during spontaneous laying.

Previous experiments (Ram, 1977) showed that the *Busycon* capsule-laying substance is protease-sensitive and, therefore, likely to be a peptide, a result also demonstrated for the egg-laying hormone of *Aplysia* (Toevs & Brackenbury, 1969). Gel filtration indicates a similar molecular weight of the *Busycon* capsule laying substance to *Aplysia* egg-laying hormone (4385 daltons, Chiu *et al.*, 1979); however, the substances are not identical, since cross-injection experiments failed to induce egg or capsule laying. Although one might attribute the failure to obtain laying in cross-injection experiments in one direction to insufficient dosage, failure in both directions must mean the hormones are not identical. This conclusion can be drawn because similar extracts of each caused laying in con-specifics. The amount of *Aplysia* extract used (0.5 abdominal ganglion per recipient) caused laying in three out of three *Aplysia* in the present experiments and is two to five times greater than the threshold dose of 0.1 to 0.25 abdominal ganglion observed by Toevs (1970). The amount of *Busycon* extract used (one CNS per recipient) caused laying of egg capsules when injected into *Busycon* (Ram, 1977) and is four to sixteen times the threshold dose of $^1/_{16}$ to ¼ CNS observed in this paper. One could not have both more than enough of the hormone to obtain laying with conspecific injections into one species and insufficient amount for cross-injection into the other and simultaneously have more than enough of the *identical* hormone in the extracts of the other species and yet not enough when injected into the first species. This result is similar to observations on cross-injection experiments between *Aplysia* and *Pleurobranchaea* (Ram *et al.*, 1977) since the *Pleurobranchaea* egg-laying hormone is also similar in gel filtration behavior, yet it, too, fails to induce laying in *Aplysia*, and *vice versa*, in cross-injection experiments.

In contrast, *Strombus* CNS caused capsule laying in *Busycon*. In previous studies on interspecific induction of egg-laying behavior, cross-injection was successful between species in the same genus (Toevs, 1970) or in the same order (Ram, 1982b), but not between species in two different orders of Opisthobranchia (Ram, *et al.*, 1977; Ram, 1982b). Similarly, galactogenin (Goudsmit and Ram, 1982) is active between different species of *Helix* but not between species in the two orders of Pulmonata (Goudsmit, E. M., Oakland University, personal communication). Both *Strombus* and *Busycon* are in the subclass Prosobranchia; however, *Busycon* is in the order Neogastropoda, whereas *Strombus* is in the order Mesogastropoda. Thus, induction of capsule laying in *Busycon* by *Strombus* CNS is the first demonstration in gastropods of inter-order induction of a reproductive response controlled by a neuronal substance. This result may imply either a closer evolutionary relationship between neogastropods and mesogastropods than has been demonstrated between other gastropod orders, or that there are atypical constraints on the evolution of the substance causing egg-capsule laying in prosobranchs. Purification, sequencing, and comparison of the substances involved, as has been done for *Aplysia* egg-laying hormone (Chiu *et al.*, 1979), would be a start in unraveling these molecular aspects of evolutionary relationships of gastropods.

ACKNOWLEDGMENTS

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LARVAL RELEASE RHYTHM COINCIDING WITH SOLAR DAY AND TIDAL CYCLES IN THE TERRESTRIAL CRAB *SESARMA*— HARMONY WITH THE SEMILUNAR TIMING AND ITS ADAPTIVE SIGNIFICANCE

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ABSTRACT

This field study determined which cycle of a lunar day or tide coincides with the time of day of larval release in the terrestrial crab, *Sesarma haematocheir.* Observations of larval release were made at a riverside 100 m upriver from Kasaoka Bay in the Inland Sea of Japan where the tidal phase differs by several hours from that of the Pacific Ocean. The findings demonstrated that the timing of larval release coincided not with a lunar day cycle but with a local tidal cycle. The larval release pattern of the Kasaoka population showed a relatively strong correlation with tides when compared with the Izu population. This suggests that the Izu population pattern was transitional, going from a combined solar day and tidal pattern to a complete daily rhythm. The timing of incubation and larval release may be based on the following mechanisms: semilunar timing of incubation entrained by lunar cycle, and the time of day of larval release controlled by a combination of solar day and local tidal cycle. This study presents further evidence that the semilunar rhythm of incubation and larval release plays an important role in the survival of larvae.

INTRODUCTION

In intertidal zones and estuaries, tidal cycles as well as light and temperature are important physical parameters. The ebb and flow of tides periodically cause drastic changes in the environment, *e.g.* fluctuations of salinity and emersion and submersion of mud flats. These changes, interacting with daily light-dark cycles and seasons, produce complex temporal situations. Under such conditions, each species evolves the means for adequate adaptation for survival and reproduction. Accordingly, behavior and physiology of many organisms inhabiting intertidal and estuarine environments correspond to predictable environmental fluctuations: the daily light-dark cycle, lunar and tidal phases, and seasons, thus showing cyclicity. Also, although considerable information has accumulated on semi-monthly cycles of reproductive activities (Korringa, 1947; Naylor, 1976), little is known about timing mechanisms except for a semilunar rhythm of emergence in the intertidal midge *Clunio* (Neumann, 1966, 1976, 1978).

The purpose of this study is to elucidate the complex timing of egg production and larval release in the terrestrial crab *Sesarma haematocheir.* An overt semilunar hatching rhythm in this species has already been reported (Saigusa, 1980b, 1981). From its habitat, it is supposed that this semilunar rhythm might be induced by a lunar cycle; thus, male and female crabs were exposed to a simulated lunar cycle

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consisting of a 24 h light-dark cycle and a 24.8 h artificial moonlight cycle in the laboratory. This treatment brought about a semilunar rhythm of incubation and larval release (Saigusa, 1980a). The question thus arises: is the time of day of larval release also correlated with the lunar cycle? (If so, it probably corresponds to a range of moonrise time extending from full moon to the last quarter and moonset time from a new moon to the first quarter.) In other words, is the larval release pattern observed in the field (Saigusa, 1980b, 1981) a combination of a 24 h solar day and 24.8 h lunar day or a combination of a solar day and a 24.8 h unimodal tidal component?

A few investigators have presented evidence that the fiddler crab *Uca* also exhibits a semilunar rhythm of larval release (Christy, 1978; Wheeler, 1978). It

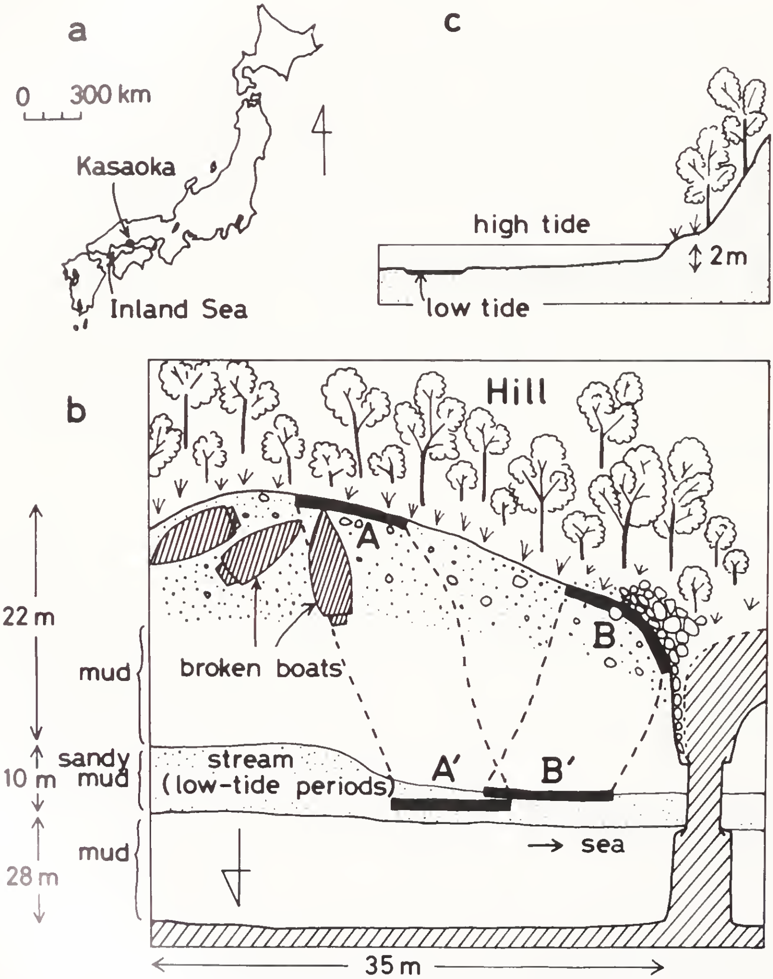

FIGURE 1. Larval release by *Sesarma haematocheir* females was observed at a point 100 m upriver from the Kasaoka Bay in the Inland Sea. Figure 1a shows the location where field work was carried out, and Figure 1b, the environment of the study area. From 11 July to 1 August 1980, site A–A′ was used for observation, and site B–B′, from 1 to 17 September 1980. Figure 1c shows a cross section of the study area.

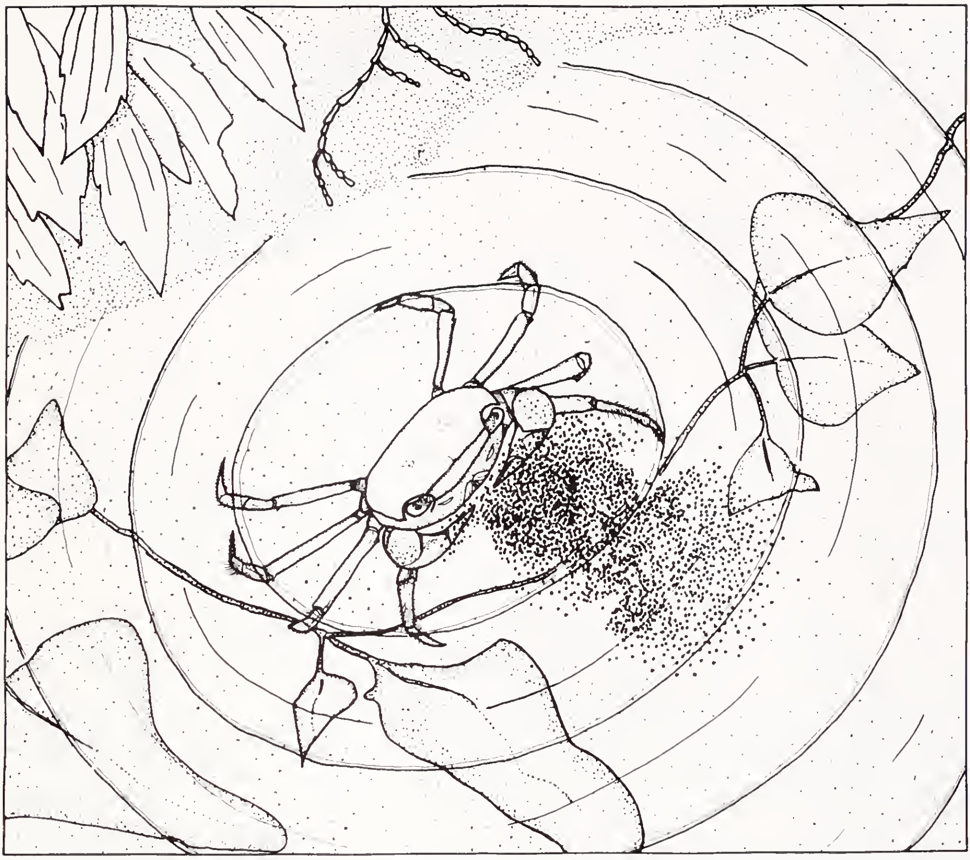

FIGURE 2. *S. haematocheir* female behavior following larval release. On the final day of embryonic development, egg laden females appear on the riverside to release their clutches at the water's edge. Holding onto stones or rocks, the females vigorously vibrate their abdomens, causing the egg membranes to break, and newly hatched zoeae to emerge.

might be worth while to look for adaptive meaning of such behavior as biological rhythm in *Uca* and *Sesarma* spp. Based on field observations of time of larval release by *S. haematocheir, Sesarma intermedium*, and *Sesarma dehaani* and experiments on the tolerance of zoeae to fresh water, concluded that the semilunar rhythm of larval release gives larvae the best chance for survival (Saigusa, 1981).

The most important point supporting this conclusion is that larval release is concentrated within a few hours after high water occurring about dusk. This poses a problem of tidal synchrony. On coasts with semidiurnal tides, the times of high and low waters on days of full and new moons have occurred at nearly the same time of day for years, but differed depending on the seashore location. (For example, on coasts of the Izu peninsula, the time is around sunrise and sunset for spring tides, but from five to six hours later in the Inland Sea.) Thus the question arises as to whether timing of larval release coincides with local tidal cycles.

The two questions above may be condensed into the following problem: is the time of day of larval release correlated with tidal cycles? To solve this problem,

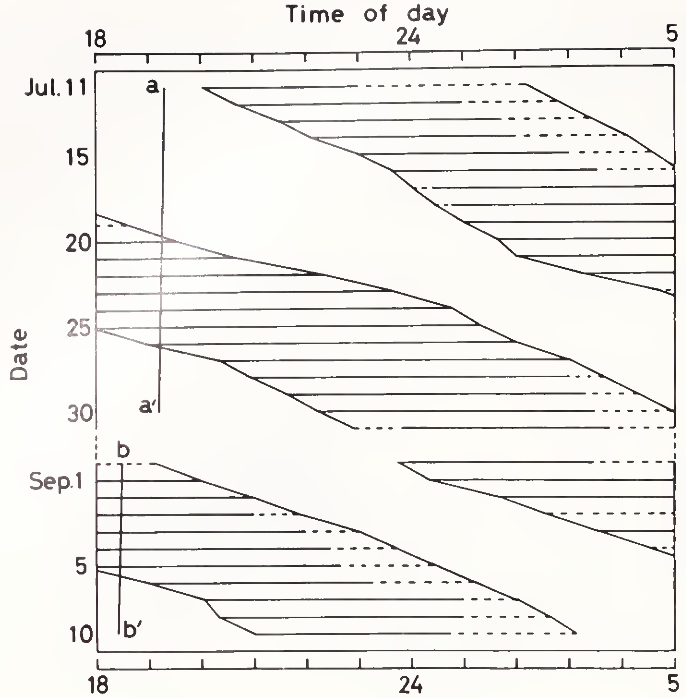

FIGURE 3. Temporal patterns of emersion and submersion on the mud flat of the study area at Kasaoka. Solid lines indicate the time from the beginning to the end of tidal influence. Dotted lines show periods at which tidal data were not obtained. Lines *aa′* and *bb′* connect the sunset times.

a river flowing into the Inland Sea was chosen for observation. On the coasts of the Pacific Ocean including the Izu peninsula, the time of moonrise and moonset is close to that of high water, so that it is very difficult to determine whether the time of day of larval release coincides with the lunar day or tidal cycle.

This paper reports the larval release rhythm of *S. haematocheir* observed at Kasaoka, Okayama Prefecture, Japan. The causes are presented for the difference in the larval release patterns between the Kasaoka population and the Izu population, along along with the timing mechanism of incubation and larval release. The relation of larval release rhythm to local tidal conditions is new evidence for the adaptive significance of semilunar reproductive rhythm in estuarine crabs.

MATERIALS AND METHODS

Larval release by *Sesarma haematocheir* females was observed in Kasaoka, Okayama Prefecture, from 11 July to 1 August, and from 31 August to 17 September 1980. A riverside area located about 100 m upriver from the Inland Sea was chosen as the study site (Fig. 1). The number of females releasing larvae was counted by an electric torch with six 1.5 V batteries. From 11 July to 1 August, a 7–8 m range along the water's edge (site A–A′) was observed, but from 31 August to 17 September, about a 10 m range (site B–B′) was used. Because a boat at A–A′ often hindered observation, the site was moved somewhat in September.

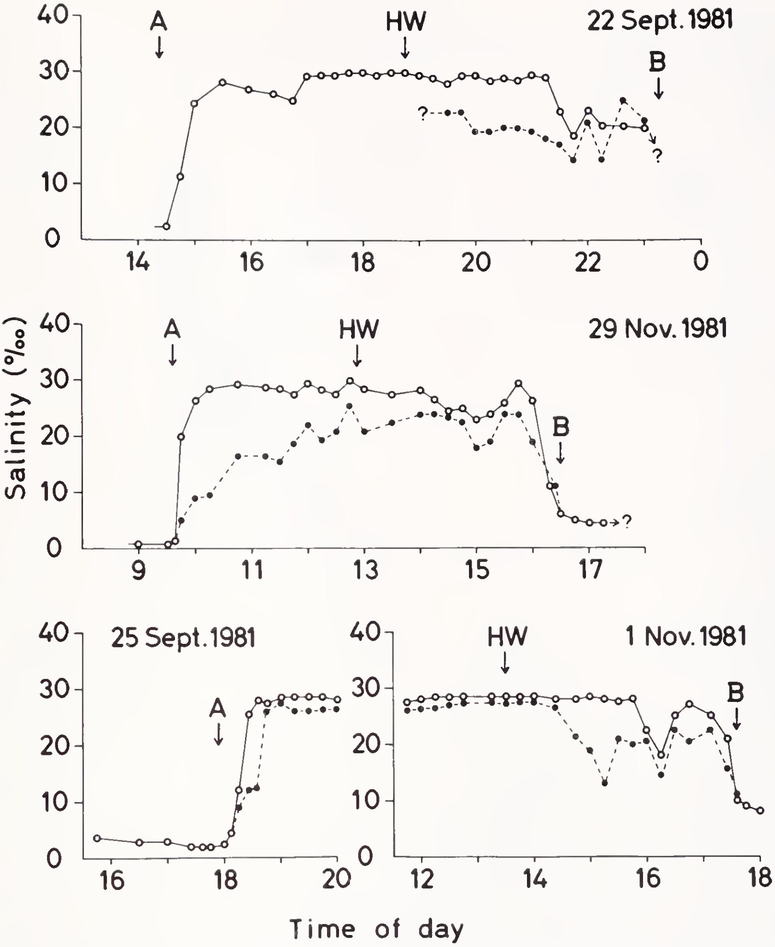

FIGURE 4. Periodic fluctuations of salinity coinciding with tidal cycles at Kasaoka. *A* and *B* show the beginning of a rising tide and the end of a receding tide, respectively. *HW* indicates the time of high water. The upper diagram gives data for the neap tide, the middle diagram data around the spring tide, and the lower diagram data for the spring and neap tides. Dark circles represent salinity at the water's edge (*i.e.*, the surface of water), and open circles indicate the salinity of the river flat at a few meters from the water's edge.

Most females released larvae at the water's edge at either the A or B site. However, within one or two days near the first or last quarter of the moon, the larvae were released at low water or at the receding tide in the middle part of the river.

Most females released larvae while holding onto stones at the water's edge (Fig. 2), but some entered the water to release them on the submerged mud flat. The water was not clear and prevented direct observation of larval release by the females which had moved into deep water. These females returned to the water's edge following larval release and thus were included in the number of females releasing larvae.

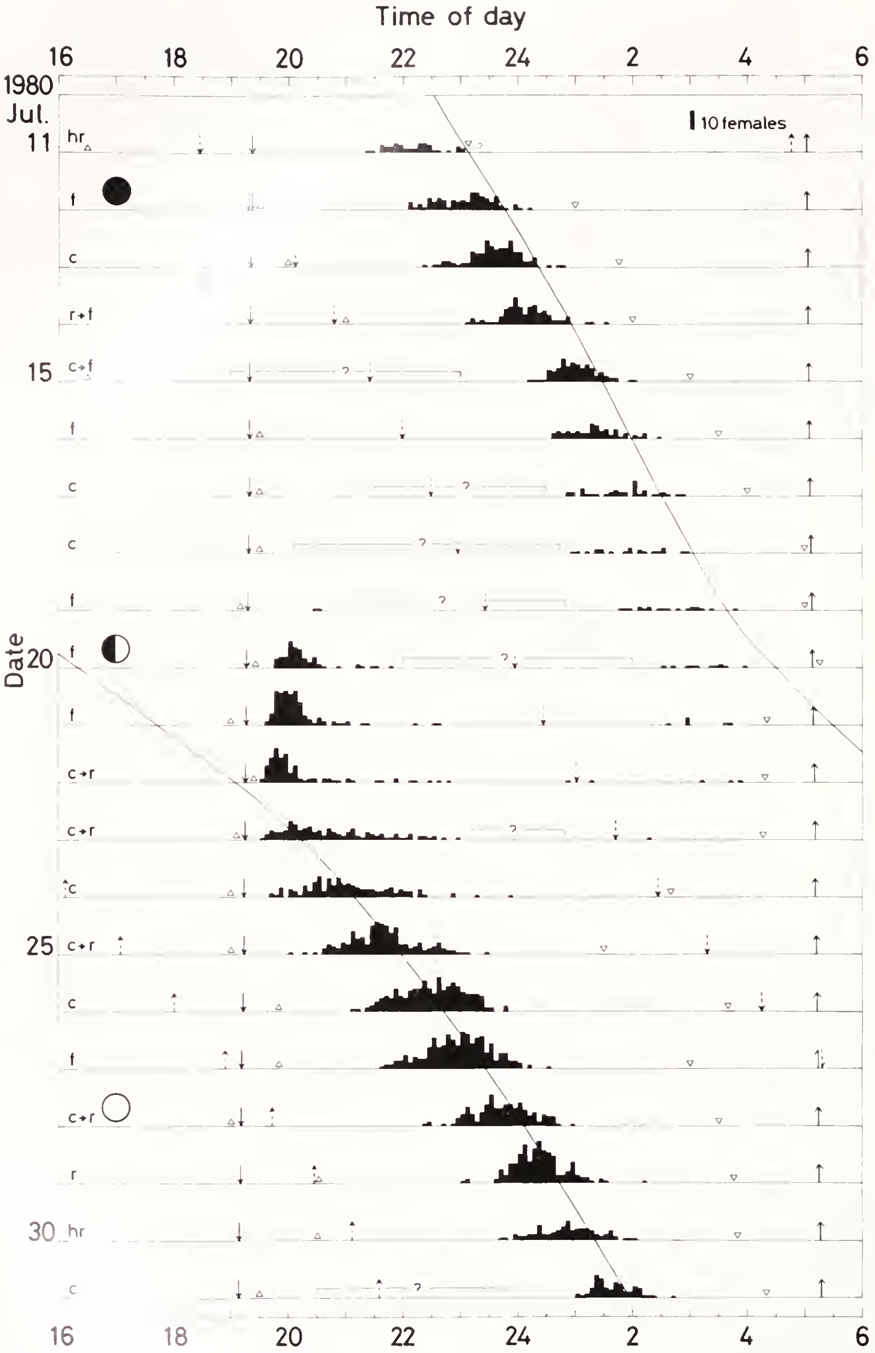

FIGURE 5(A): *S. haematocheir* larval release rhythm at Kasaoka 11 July to 1 August 1980. The number of females releasing larvae per five minutes is expressed in the data. The upward and downward solid arrows represent sunrise and sunset times, respectively. The upward and downward broken arrows show moonrise and moonset times, respectively. The solid diagonal lines connect the high water times, *i.e.*, when the river reached maximum depth. The tidal data were based mostly on the survey, and partly on the tide table. Weather during the observations: fine (f), cloudy (c), rain (r), heavy rain (hr). Question

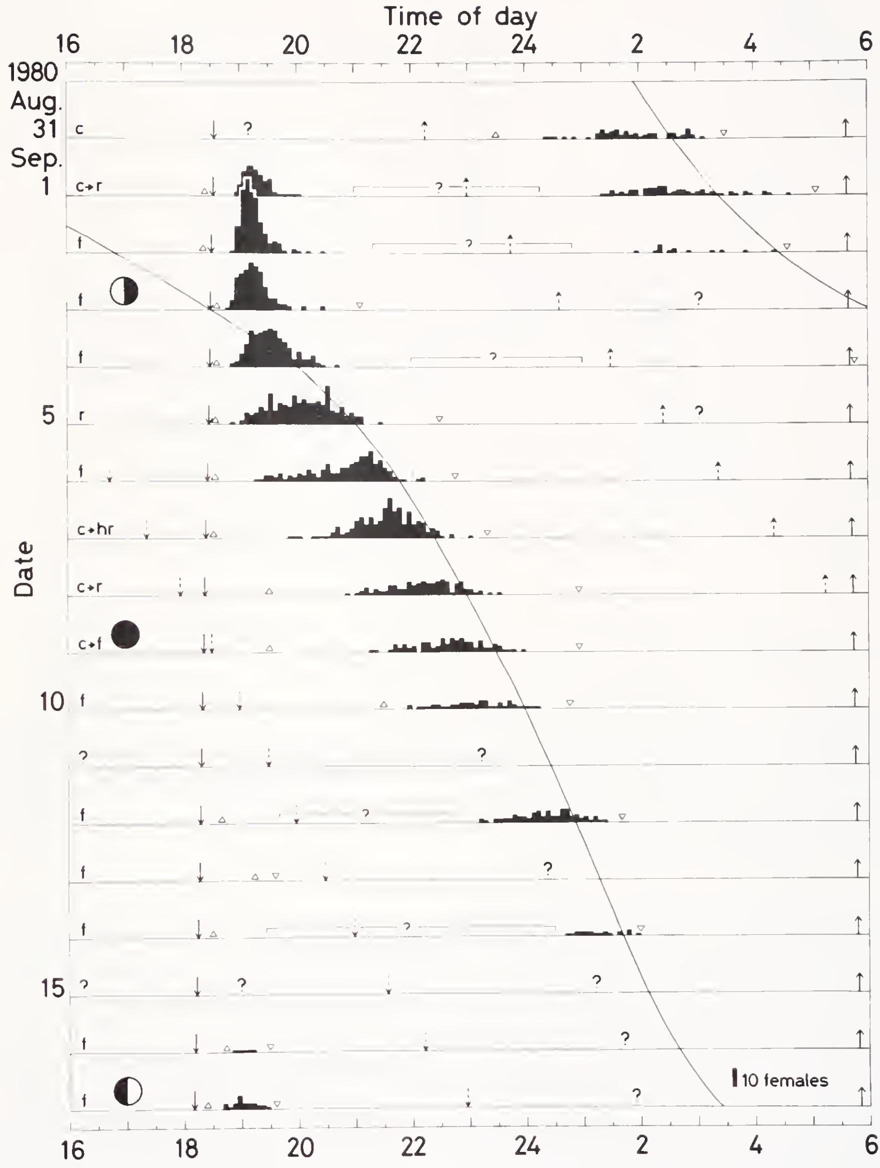

FIGURE 5(B): *S. haematocheir* larval release rhythm at Kasaoka, 31 August–17 September 1980. Symbols are the same as in Figure 5A.

RESULTS

Tidal conditions at Kasaoka

The time of sunrise and sunset on the Inland Sea comes only 18 ± 1 min later everyday, but the tidal phase comes 5–6 h later when compared with the coasts

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marks indicate the day or time when observations could not be made. Beginning and end of observations are shown by upward open triangles (△) and downward open triangles (▽), respectively. The dark and open circles represent new and full moons, respectively. Semicircles, the first or last quarters of the moon.

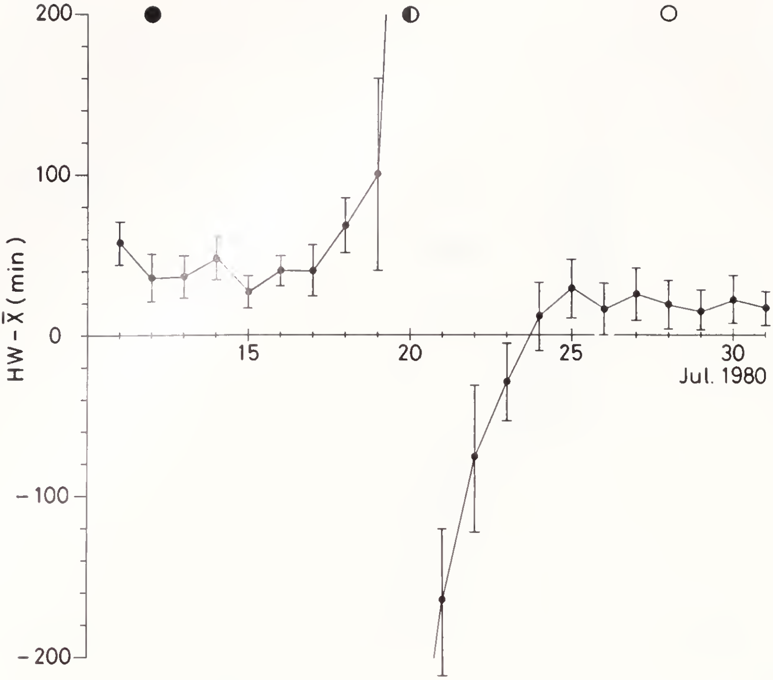

FIGURE 6(A): Correlation between the timing of larval release by the Kasaoka population and the time of high water, 11–31 July 1980. The estimated time from the average peak of the larval release activity ($\bar{X}$) to the time of high water (HW) is plotted. *HW* indicates the time of high water with which larval release coincided. Temporal variation in larval release activity is indicated by standard deviation. Though a nighttime period virtually extended two days, larval release from 24:00 till dawn is included on the date preceeding the actual date.

of the Izu Peninsula. Thus, high water in Kasaoka occurs about noon and midnight at the spring tides and around sunrise and sunset at the neap tides.

In this study site, the river flat emerged at the low water periods, when the river became 5–6 m wider and about 5 cm deeper. The entire estuarine flat was submerged during the high water periods, when the maximum depth of the middle part of the river was recorded at night to be 2.5 m in July and 2.2 m in September. Although few measurements were made of the daytime high water, the tide table indicated that the water level during summer was always lower at the daytime high water than at the nighttime high water, and that the difference between them was 30–50 cm at the spring tides. The water level at the neap tides was recorded at 1.5–1.8 m in the morning and evening high-water periods. Though some difference could be seen in the water level at the high water periods according to the season and lunar phase, it had relatively little influence on the period of emersion and submersion of the mud flat (Fig. 3).

Salinity changed remarkably with ebb and flow of the tides. Also, at high water periods, a considerable difference in salinity was often recorded between water edge or surface and a place where the water was somewhat deeper. The periodic fluctuations in salinity showed a similar pattern in spring and neap tides (Fig. 4).

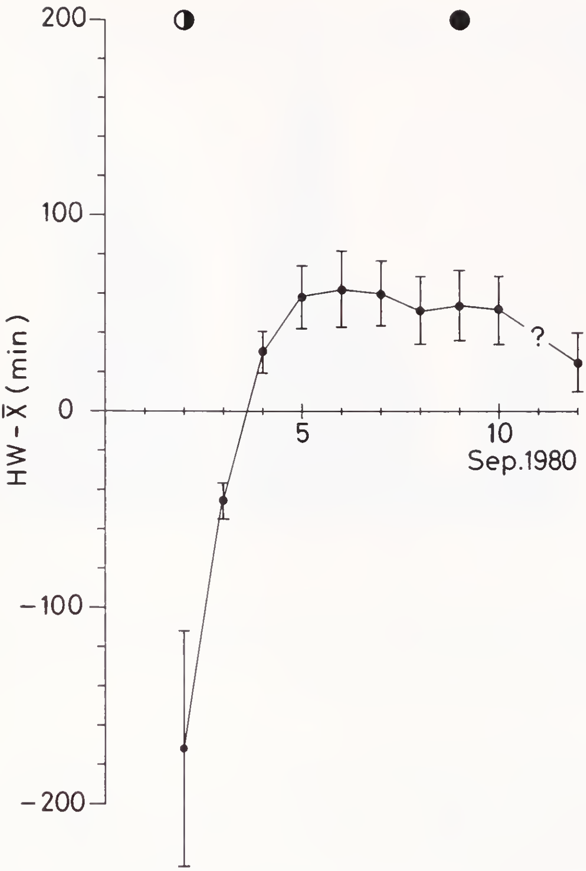

FIGURE 6(B): Correlation between the timing of larval release by the Kasaoka population and the time of high water, 1–12 September 1980. The value for HW-X̄ was calculated by the same method shown in Figure 6A.

Semilunar rhythm of larval release at Kasaoka

The time of day of larval release at Kasaoka is shown in Figure 5. It is evident that the time of day of larval release did not coincide with a lunar day cycle, but with a local tidal cycle. Larvae were released only at night, suggesting that the solar day cycle suppressed daytime larval release in all cases. In addition, larval release activity revealed a combined pattern of 24 h solar day and 24.8 h unimodal tidal components, corresponding to the phase relationship between a solar day cycle and 12.4 h tidal cycle. The solar day component appeared when high water came about sunset and sunrise, and the tidal component appeared at different times.

When the larval release activity revealed a strong correlation with nighttime high water, the average activity peak (X̄) was a little before the high water (HW) (Fig. 6A, B). The estimated time from X̄ to HW then differed somewhat in tidal cycles during the night: 42 min from 11 to 17 July, 17 min from 24 to 31 July, and 49 min from 4 to 12 September, respectively on the average. The average peak

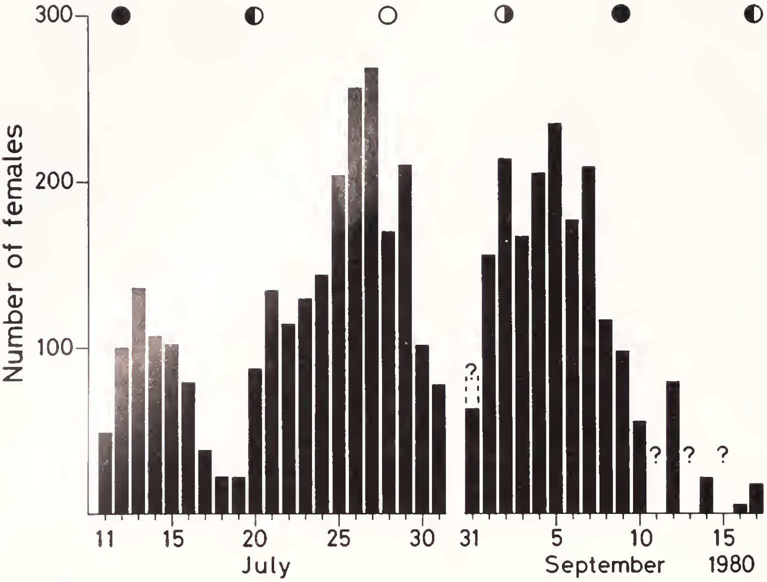

FIGURE 7. Fluctuation in the number of females releasing larvae during the night 11–31 July and 31 August–17 September 1980. Females releasing larvae from 24:00 till dawn are included on the date preceeding the actual date. The open circle indicates a full moon, the dark circles, new moon, and the semicircles, the first and last quarters of the moon.

showed less correlation with tides as the time for high waters approached dawn and sunset, *e.g.* 18 to 20 July (Fig. 6A).

Fluctuation in the number of females releasing larvae every night is shown in Figure 7. Larval release activity in July was semilunar, peaking on the day following the new moon on 12 July and on the day before the full moon on 28 July. More females were seen during the several days close to the full moon than during the new moon. At about the last quarter in September, many females released larvae just after sunset (Fig. 5B), so that larval release activity in September was higher near the last quarter than near the new moon with respect to the lunar phase.

Comparison of larval release patterns between the Kasaoka and Izu populations

Figure 8 shows the *S. haematocheir* larval release rhythm at the Izu peninsula (data from 8 to 24 September 1976). The most remarkable difference between the Kasaoka and Izu population patterns (Figs. 5A, B) is the correlation with the nighttime high water: while the Izu population larval release correlated only weakly with the high water for several days (Fig. 9), the Kasaoka population larval release had a relatively strong correlation with it.

Another difference is the variation in timing of larval release according to the phase relationship between solar day and tidal cycles. While standard deviation in the Izu population activity was very small at times when the afternoon high water came 1–3 h before sunset, that of the Kasaoka population was quite large. In contrast to this, standard deviation in the Izu population timing gradually increased as the high water on one occasion approached midnight, while that of the Kasaoka population was about constant.

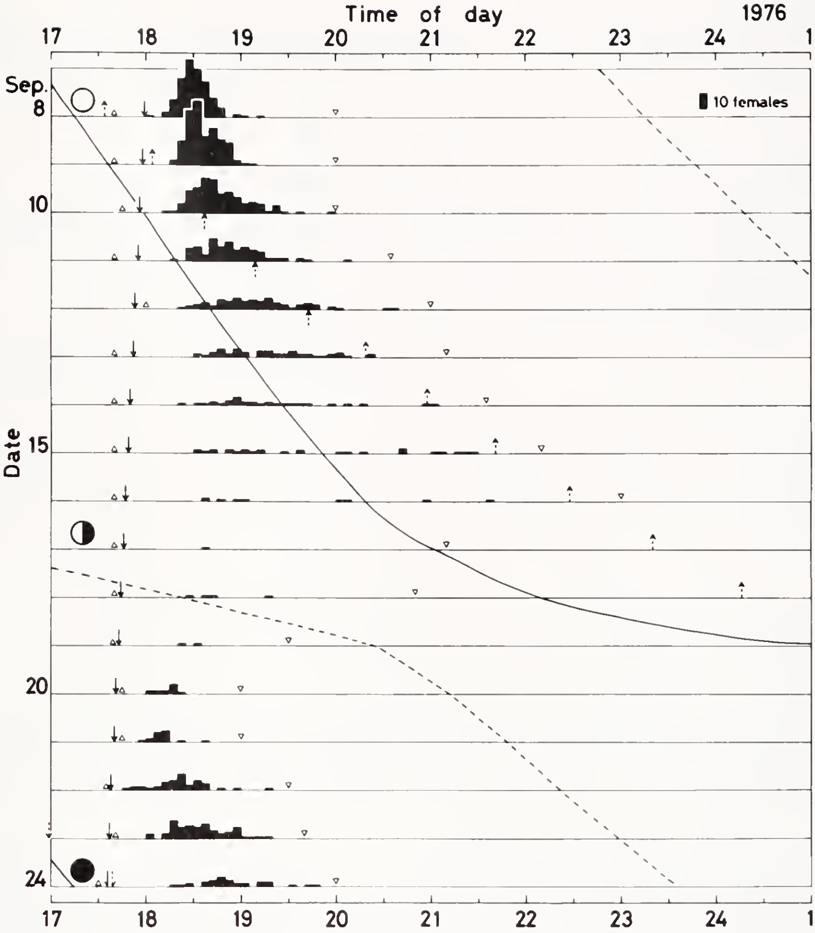

FIGURE 8. *S. haematocheir* larval release rhythm observed at a point 1.5 km upriver from the Pacific Ocean, 8–24 September 1976. The solid and broken diagonal lines connect the times of high water and low water occurring on the seacoast, respectively. These times are not based on the survey but on the tide table in 1976. Other symbols are the same as in Figure 5A.

Standard deviation in each local population was considerably smaller at syzygy, and became larger around a half moon. This is interpreted as an apparent correspondence to the lunar phase, since tidal phase at Kasaoka shifted about 180°, compared with the Pacific Ocean.

DISCUSSION

Larval release rhythm with solar day and unimodal tidal components

For organisms having a semilunar reproductive periodicity, the time of day of reproduction may be divided into two periods with respect to environmental cycles: timing coinciding with a solar day cycle, *e.g.* emergence of the intertidal midge *Clunio Marinus* (Neumann, 1966, 1976) and the sublittoral midge *C. balticus* (Heimbach, 1978), and timing coinciding with either semidiurnal tide, *e.g.*

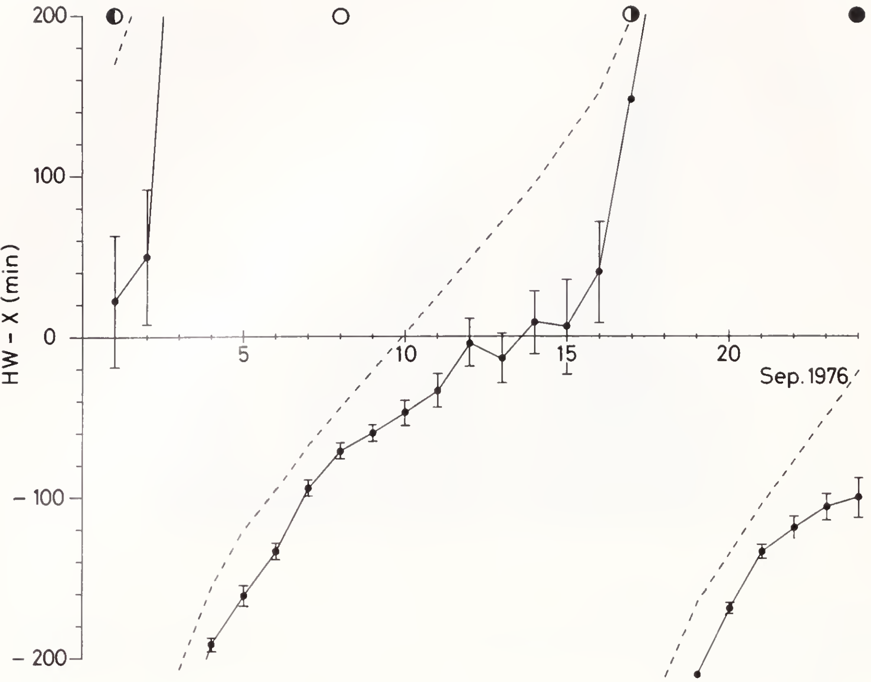

FIGURE 9. Correlation of the timing of the Izu population larval release with high water 1–24 September 1976. The value for HW-X̄ was calculated by the same method shown in Figure 6A. Made from data published elsewhere and Figure 8. Broken lines connect the estimated time from sunset to the high water.

the Japanese midge *C. tsushimensis* (Oka and Hashimoto, 1959) and the grunion *Leuresthes tenuis* (Enright, 1975). In addition, timing coinciding with both solar day and tidal cycles in the *Sesarma* larval release rhythm is reported here.

The combined effects of solar day and tidal components have been described for locomotor activity rhythms of several species of crabs, *e.g. Carcinus* (Naylor, 1958), *Sesarma* (Palmer, 1967) and *Uca* (Barnwell, 1966; Honegger, 1976; Webb, 1976). Whereas locomotor activity usually shows a bimodal tidal component, most reproductive activity seems to be correlated with either semidiurnal tide.

The tidal component was much stronger in the Kasaoka (Figs. 5A, B) than in the Izu population pattern (Fig. 8). This suggests that in the Izu population pattern, the tidal component diminishes. This may be transitional, going from a combined solar day and tidal pattern to a completely daily rhythm.

Two possibilities for the difference in both population patterns are: a direct response to a certain difference in tidal conditions surrounding each population, and population-specific rhythm. Although the study site at the Izu Peninsula was situated at a point near the limit of where tidal amplitude affected the water level, the site at Kasaoka was located only 100 m from the sea. Various influences produced by the tidal cycle would be considerably weakened at a site (Izu) 1.5 km from the seacoast. (For example, tidally correlated vibration would be extremely lessened; and, tidal amplitude might effect the water level only near spring tides.) Therefore, one possibility is that the difference in larval release patterns results from a direct response of females to differences in tidal conditions. Another possibility is that the release is based on population-specific properties. If so, each *S. haematocheir* population would show the same pattern as observed here, at any river site inhabited by this population.

Timing mechanism of egg production and larval release

According to Neumann (1966, 1976), the midge *C. marinus* from southern and middle Europe emerges near daytime low water during a few days at spring tides. He kept cultures of the midge in a 24 h light-dark cycle and then exposed them to a faint light of 0.4 lux a few days each month. This treatment evoked a semilunar rhythm of emergence. In addition, the time of day of emergence followed a 24 h light-dark cycle. He thus demonstrated that the emergence of *C. marinus* is based on a combination of two different timing mechanisms: a semilunar timing of pupation and a daily timing of emergence.

S. haematocheir males and females were exposed to a simulated lunar cycle (Saigusa, 1980a). At first egg production the crabs were divided into three groups: two groups synchronizing with the artificial new moon and one with the artificial full moon (Fig. 7 in *Oecologia* Vol. 46, p. 41). The time from the start of incubation to larval release was about 30 days for each individual; larval release was semilunar. Since nearly all females started the second incubation within a few days, the second larval release also showed semilunar rhythm.

This may be relevant to the field data obtained at the Izu peninusula (Fig. 10): the semilunar rhythm of larval release in the Izu population may also correspond to the three groups of females, *i.e.* two coinciding with the full moon (G-1 and G-3) and one with the new moon (G-2). The peak of larval release on 16 July would have corresponded to the group, G-1, which started egg production near full moon in June. This group would have soon started the second egg production, showing a peak of larval release after 30 days, *i.e.* on 15 August. Moreover, some females might have started the third incubation and released larvae near full moon in September. Another group, G-3, would have started the first incubation near full moon in July, so that the peak of the larval release would have occurred in the next month, on 11 August. This group would have started the second egg production soon, and the peak on 8 September would have corresponded to this group releasing the second clutch. Moreover, another group, G-2, with incubation and larval release coinciding with new moons, would have existed.

This explanation, however, does not account for the inexact correspondence of the semilunar rhythm of larval release to lunar phase. For example, (Fig. 10), though four peaks from August to September have good correspondence with syzygy, peaks on 16 July, 30 July, and 15 August occurred several days after the full and new moons. The Kasaoka population larval release also showed a peak several days before the new moon in September (Fig. 7). These findings might arise from temperature dependency on the period of incubation, or delay or advance of the beginning of incubation from syzygy.

On the other hand, this study presents evidence that the main factors controlling each population's larval release pattern are solar day and local tidal cycles, not the lunar phase. Thus, the timing mechanism of egg production and larval release is interpreted in terms of the following: semilunar timing of incubation and larval release and the time of day of larval release controlled by solar day and tidal cycles.

Adaptive significance of semilunar reproductive rhythm

There are several possibilities for the adaptive function of the semilunar reproductive rhythm exhibited by the fiddler crab *Uca*. For instance, tidal currents

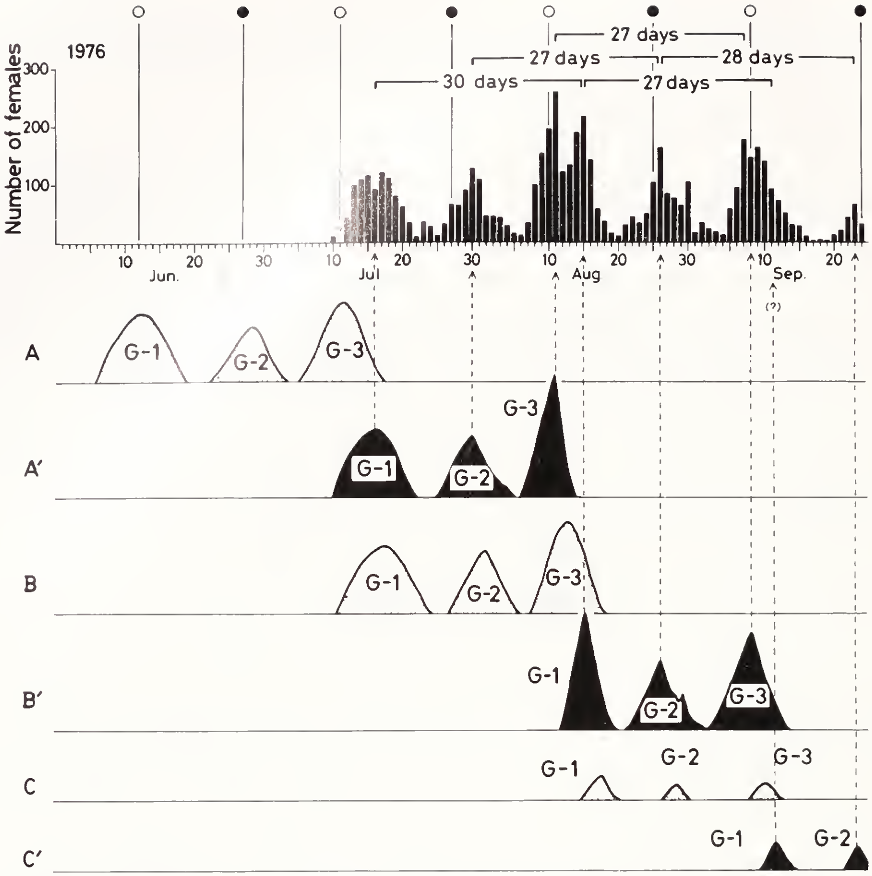

FIGURE 10. Construction of a semilunar larval release rhythm observed at the Izu peninsula in 1976. Results in the laboratory apply to the field data. *A*, the first egg production; *A'*, the first larval release; *B*, the second egg production; *B'*, the second larval release; *C*, the third egg production; *C'*, the third larval release. For further explanation, see text.

associated with high water around full and new moons disperse larvae most effectively in the sea (Wheeler, 1978), and this provides the best opportunity for megalops to be transported to substrates suitable for adults (Christy, 1978; Zucker, 1978).

According to Vernberg and Vernberg (1975), fiddler crab larvae have little tolerance for reduced salinity, though species and seasonal differences exist. The tolerance of *S. haematocheir* and *S. intermedium* zoeae for fresh water is very low, though they are much more resistant of low salinity than *Uca* zoeae. These facts suggest that zoeae released into the river under extremely low salinity conditions might perish.

Salinity at Kasaoka rapidly increased with rising tide, becoming a maximum for a period of six hours when the water was flowing. A comparison of Figure 4 with Figures 5A and B suggests that *S. haematocheir* zoeae are released into the tiver when the salinity is most favorable: on 20 July (Fig. 5A) and 1 September (Fig. 5B), larvae, released just after sunset, have been exposed to extremely low salinity. At other times, the receding tides carry the larvae to the sea very effectively. Thus, the relation of the semilunar rhythm of larval release to local tidal conditions enhances the probability of larval survival.

For organisms showing semilunar reproductive rhythm, the time of day of reproduction is correlated with either the daytime or nighttime tide. For example, *C. tsushimensis* emerges at the time of daytime low water in summer and nighttime low water in winter (Oka and Hashimoto, 1959). Spawning by *L. tenuis* occurs at high water after sunset (Enright, 1975). *Uca* females apparently release zoeae near high water (DeCoursey, 1979). Larval release by three species of *Sesarma* also occurred at nighttime high water (Saigusa, 1981).

DeCoursey (1979) has suggested that egg hatching at nocturnal high water may minimize predation on egg laden *Uca* females walking to the water's edge to release larvae and also reduce predation on larvae. A special predator on adult and young *Sesarma* is not known as yet. However, schools of the grey mullet (*Mugil cephalus*) were observed entering the rivermouth at rising tide and busily consuming newly released *Sesarma* larvae at the water's edge. This suggests predatory pressure upon larvae at nighttime high water.

The adaptive significance of nighttime larval release by crabs inhabiting estuaries, is obscure. Yet, egg laden females might walk to the riverside by sunset and release larvae.

ACKNOWLEDGMENTS

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STYLET FORMATION IN NEMERTEANS

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Abstract

Stylet formation was examined in nine species of nemerteans by light microscopy. The first stylets produced by larvae are assembled intracellularly over a period of several days within styletocytes of reserve stylet sacs. A reserve stylet is moved to the lumen of the proboscis, apparently by muscular contractions, and placed on the basis to become the central stylet. The stylets of adult nemerteans are also formed in styletocytes of reserve stylet sacs, and, depending on the species, reach full length in two to eight weeks. At the onset of styletogenesis, a membrane-bound vacuole develops in the styletocyte, and an organic matrix is formed at one edge of the vacuole. The calcified cortex of the stylet shaft is subsequently deposited around the organic matrix, and a knob-shaped proximal piece is formed on the shaft. Most adult nemerteans contain at least one developing stylet, and the rate of stylet formation is about the same in starved worms as it is in worms that have recently captured prey. Replacement of the central stylet occurs following prey attack, and occasionally when the worm is not feeding.

Introduction

Nemerteans belonging to the order Hoplonemertea typically possess an eversible proboscis that is armed with a calcareous central stylet. During prey capture, the central stylet is used to inflict wounds, into which paralytic neurotoxins are introduced (Kem, 1973; Stricker and Cloney, 1981). In addition to the central stylet, the proboscis usually contains two to several reserve stylet sacs, in which reserve stylets are formed. Reserve stylets are believed to replace the central stylet, when it becomes damaged or lost (Gibson, 1972).

A fully developed stylet is nail-like in shape and can range in length from about 8 μm in *Carcinonemertes carcinophila* (Humes, 1942) to over 200 μm in species such as *Emplectonema gracile* (Coe, 1901). Each stylet consists of a tapered shaft and a knob-shaped proximal piece. Both regions of the stylet are composed of an inner organic matrix surrounded by an inorganic cortex. The cortex contains mainly calcium, phosphorus, and strontium (Wourms, 1976).

The first extensive studies on the formation of nemertean stylets were conducted by Bürger (1895) and Coe (1905). Recently, Wourms (1976), Stricker (1981), and Stricker and Cloney (1981) have briefly reported on the ultrastructure of styletogenesis.

In this paper, we describe the formation of stylets in nine species of hoplonemerteans. Styletogenesis in adult worms is compared to the formation of the first stylets by larvae. Replacement of the central stylet by a reserve stylet is discussed, and the time required for stylet formation is estimated for three species.

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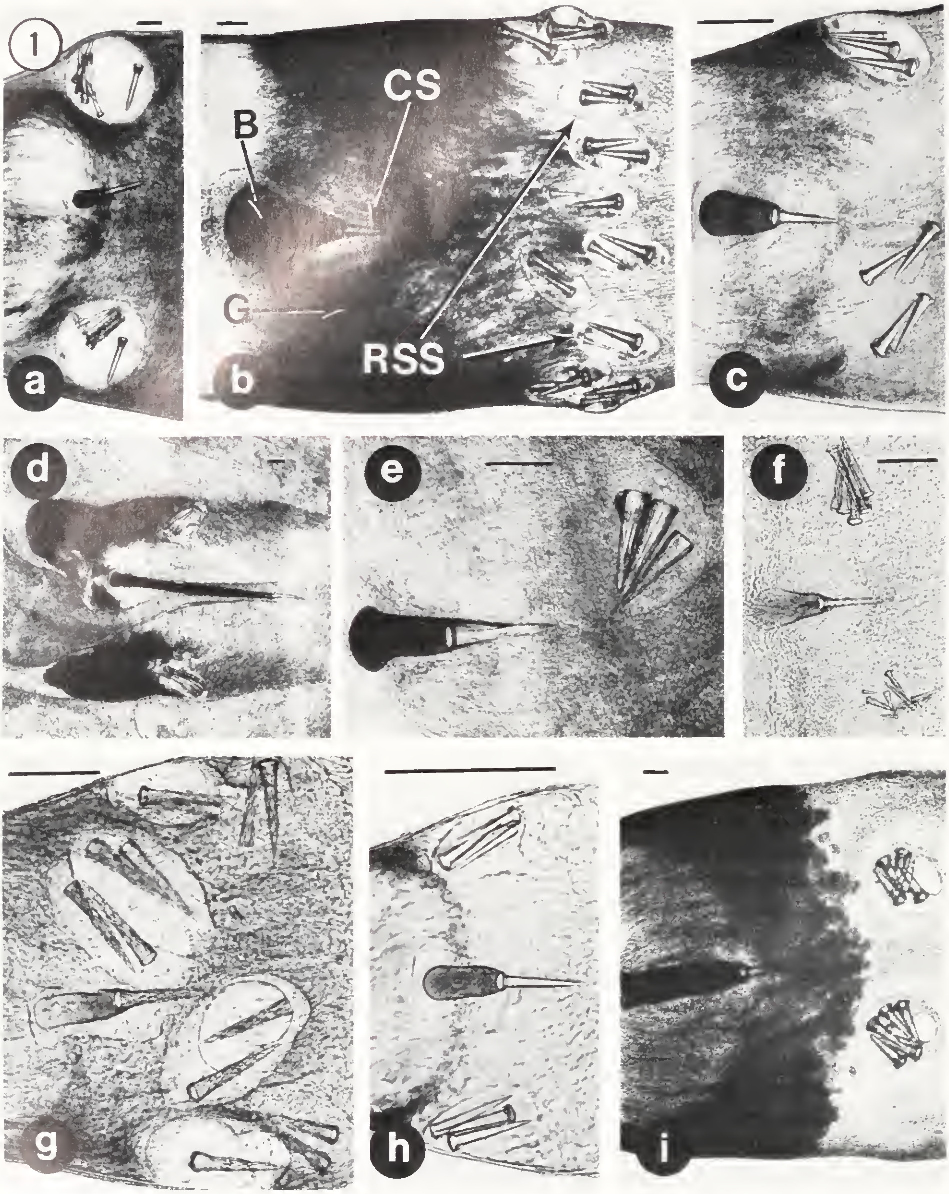


FIGURE 1. Whole mounts of the stylet apparatus in living proboscides removed from adult worms. The central stylet (CS) is attached to a granular basis (B) and surrounded by two to several reserve stylet sacs (RSS), as well as prominent glands (G). Each stylet consists of a knob-shaped proximal piece and an elongate, tapered shaft. Scale bar in 1a–i = 100 μm. a) *Amphiporus bimaculatus*, ×35; b) *A. formidabilis*, ×40; c) *A. imparispinosus*, ×100; d) *Emplectonema gracile*, ×25; e) *E. purpuratum*, ×90; f) *Paranemertes peregrina*, ×80; g) *Paranemertes* sp. ×110; h) *Tetrastemma* sp., ×185; i) *Zygonemertes virescens*, ×40. Note: Figure 1d) depicts the proboscis *in situ*, and 1e) shows only one of the two reserve stylet sacs that are present in *E. purpuratum*.

MATERIALS AND METHODS

The following nine species were examined in this study: *Amphiporus bimaculatus* Coe, 1901; *A. formidabilis* Griffin, 1898; *A. imparispinosus* Griffin, 1898;

Emplectonema gracile (Johnston) Coe, 1901; *E. purpuratum* Coe, 1905; *Paranemertes peregrina* Coe, 1901; *Paranemertes* sp.; *Tetrastemma* sp.; and, *Zygonemertes virescens* (Verrill) Montgomery, 1897. Specimens were collected intertidally on San Juan Island, Washington, or by dredging in adjacent waters. Identifications were based on descriptions presented by Coe (1901, 1905, 1940).

For general studies of styletogenesis in adult worms, whole mounts of living material were examined with a compound microscope. In this paper, the term whole mount always refers to preparations of live, unstained material. The proboscides of thick or pigmented forms were removed in order to observe the stylets, but in relatively translucent specimens, stylets were examined through the body wall.

To study the development of the stylets in larvae, gravid specimens of *Emplectonema gracile* were segregated by sex into bowls containing about 1 liter of filtered sea water, and gametes were obtained following natural spawning or spawning induced by mild shock treatment (30 volts for 5 sec; Powerstat Type 116 voltage generator). The oocytes were washed with filtered sea water and inseminated at various times with diluted suspensions of sperm. Embryos were raised in fingerbowls containing unfiltered sea water at 10–12°C and checked periodically with a compound microscope to monitor the development of the stylets. Egg masses of *Tetrastemma* sp. were collected from the field and reared in the laboratory at 10–12°C. When the stylet apparatus became visible, the worms were removed from their egg mass and observed with a compound microscope.

The rates of stylet formation were studied in three species (*Amphiporus formidabilis*, *Emplectonema gracile*, and *Tetrastemma* sp.), by examining slightly compressed, whole mounts of living, $MgCl_2$-relaxed specimens. The worms were kept in individual containers in the laboratory, and the number of stylets formed by each worm was monitored over a period of several weeks. Some specimens of *Amphiporus formidabilis* and *Tetrastemma* sp. were fed amphipods obtained from the habitat in which the worms were collected; the rest of the experimental worms were not fed during the observation period. In addition, stylets were counted in animals maintained for up to 8 weeks without food, and compared to the number found in worms freshly collected from the field.

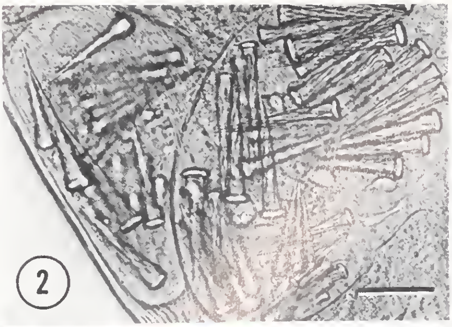
The frequency of stylet replacement was studied in *Tetrastemma* sp. following capture of three species of amphipods (*Hyale frequens*, *Paracalliopiella pratti*, and *Aoroides* sp.). The number of reserve stylets was counted in whole mounts of experimental worms prior to prey attack, and the worms were checked within one hour following capture of an amphipod, to see if the central stylet was in the process of being replaced, or if the number of reserve stylets had been reduced.

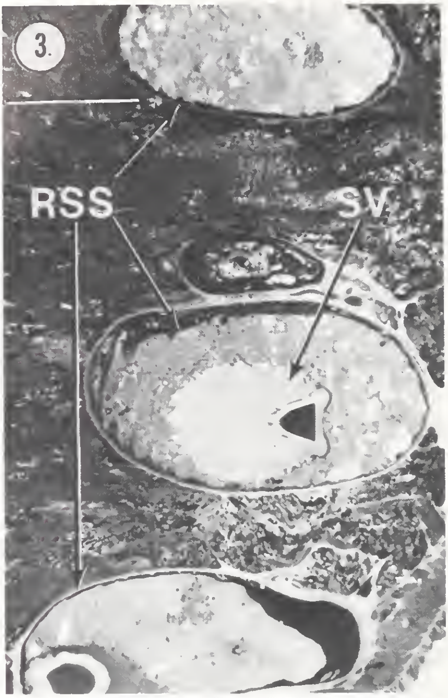
For histological studies, proboscides removed from $MgCl_2$-relaxed adults, and whole larvae with developing stylets, were fixed, decalcified, and embedded in Epon, according to methods described previously (Stricker and Cloney, 1981; Stricker and Reed, 1981). One micrometer sections were cut with glass knives and stained with a mixture of methylene blue and azure II (Richardson *et al.* 1960), or with a PAS stain, according to the method of Munger (1961).

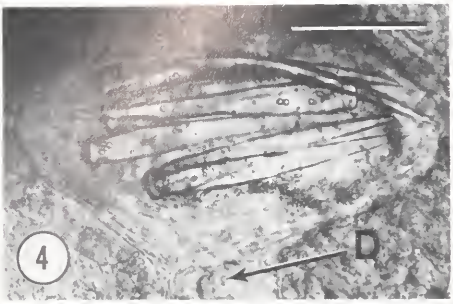
RESULTS

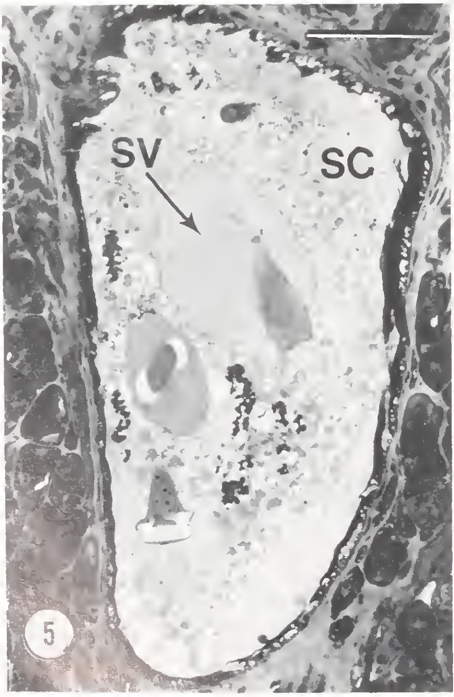
Comparative morphology of the stylet apparatus

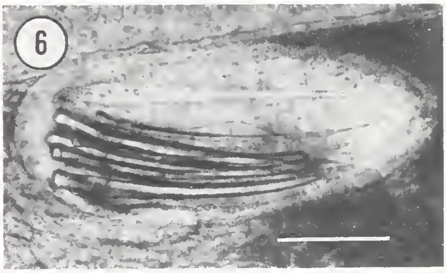
The stylet apparatus of a typical hoplonemertean is located in the mid-proboscis region and consists of a central stylet attached to a basis, and two to several reserve stylet sacs in which reserve stylets are formed (Fig. 1). In most species, the shaft of the central stylet is smooth and straight. Species such as *Emplectonema purpuratum*, *Paranemertes peregrina*, and *Paranemertes* sp., however, have helically-











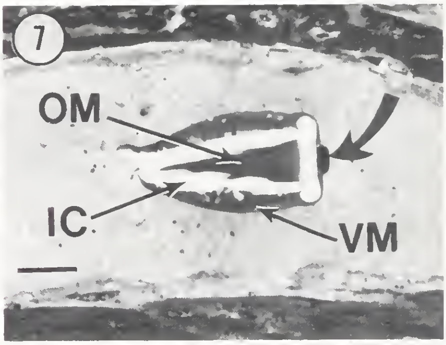


FIGURE 2. Whole mount of two reserve stylet sacs in a specimen of *Paranemertes peregrina* that had an unusually large number of reserve stylets. Reserve stylet sacs typically contain two to several reserve stylets. ×200. Scale bar = 50 μm.

FIGURE 3. Longitudinal section through three reserve stylet sacs (RSS) of *Amphiporus formidabilis*. Each sac consists of a squamous epithelium that envelops a large central cell, called the styletocyte. Stylet vacuoles (SV) are visible within the styletocytes. ×345. Scale bar = 50 μm.

wound grooves along their stylet shafts, and in *E. gracile* the stylet is markedly curved. The basis is an extracellular mass of granules that appear to be partially calcified. It is usually pyriform in shape and equal in length to the central stylet. In *Zygonemertes virescens*, however, the basis is cylindrical with a truncated posterior end, and in *E. gracile* it is much longer than the central stylet.

The reserve stylet sacs are located anterior to, or at the same level as, the central stylet. They usually contain only a few reserve stylets, but on occasion numerous stylets can be seen in a single sac (Fig. 2). The squamous epithelium that comprises the sac surrounds a large central cell, called the styletocyte (Fig. 3), and a duct extends from each reserve stylet sac to provide a pathway for the transfer of reserve stylets from the styletocyte (Fig. 4). One to several stylet vacuoles are located in the styletocyte cytoplasm (Figs. 3, 5). Each vacuole contains a vacuolar matrix that envelops a single developing stylet. Stylets in different stages of development occur in the same styletocyte, and usually do not exhibit any preferred orientation in the styletocyte. An exception occurs in *Emplectonema gracile*, where the stylets tend to be positioned with their proximal pieces facing anteriorly (Fig. 6). Fully formed reserve stylets are identical in structure to central stylets. Central stylets, however, are not located in a cell or enclosed in a vacuole. Each mature reserve stylet has a basophilic organic matrix and an outer inorganic cortex that appears as an unstained area in decalcified preparations of almost all species examined (Fig. 7). In *Amphiporus formidabilis*, however, transverse sections of decalcified stylets often reveal a thin layer of basophilic material that is arranged concentrically around the organic matrix; this layer probably represents organic matrix that is interspersed in the inorganic cortex. In many species the vacuolar matrix of a stylet vacuole contains a basophilic disc of unknown function located next to the proximal piece (Fig. 7, arrow). This disc-like structure has not been observed on any central stylet.

Stylet formation in adults

In all species that we observed, and in those examined by Bürger (1895), stylet formation follows a similar basic pattern in that the stylet shaft develops first and the proximal piece is not formed until the shaft is well developed.On rare occasions, a stylet can be seen with a proximal piece on a very short shaft (Fig. 8). It is not clear in such cases whether the shaft is in the process of actively elongating following the formation of the proximal piece, or if the stylet is fully mature but abnormally short.

During the first stage of stylet formation, a membrane-bound vacuole develops

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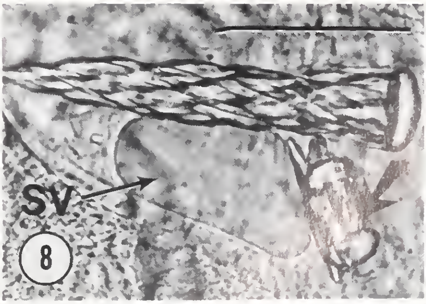
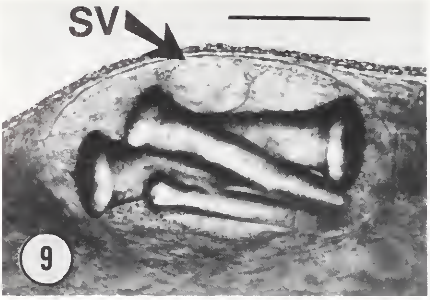
FIGURE 4. Whole mount of a reserve stylet sac of *E. gracile.* A duct (D) arises from each sac and communicates with the region around the central stylet, thus providing a pathway for the transfer of stylets. ×175. Scale bar = 100 μm.

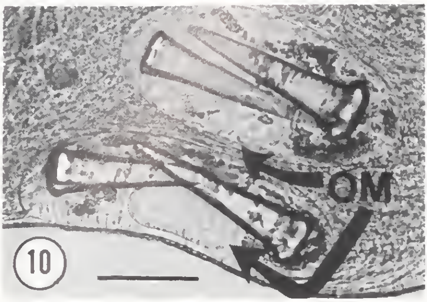
FIGURE 5. Longitudinal section through a reserve stylet sac of *Emplectonema gracile.* The styletocyte (SC) contains a weakly staining ground cytoplasm, in which several stylet vacuoles (SV) are located. ×330. Scale bar = 50 μm.

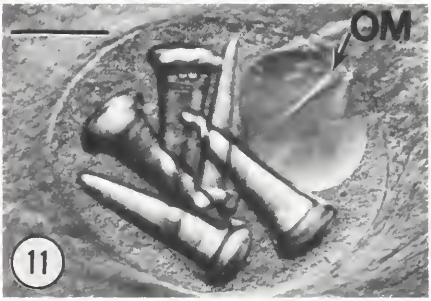
FIGURE 6. Whole mount of a reserve stylet sac of *Emplectonema gracile.* Stylets in this species tend to have their proximal pieces oriented anteriorly. In all other species examined, no preferred orientation was observed. ×150. Scale bar = 100 μm.

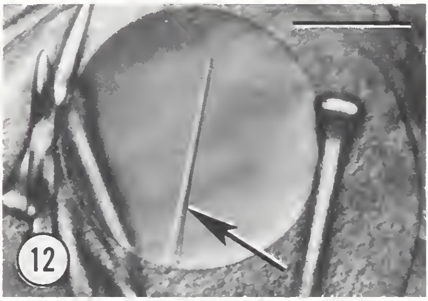
FIGURE 7. Longitudinal section through a styletocyte of *Paranemertes peregrina.* Stylets develop in vacuoles surrounded by a vacuolar matrix (VM). Mature stylets consist of an organic matrix (OM) and an inorganic cortex (IC), that appears as an unstained region in decalcified preparations. In many stylet vacuoles, a basophilic disc of unknown function can be seen next to the proximal piece of the developing stylet (arrow). ×835. Scale bar = 10 μm.

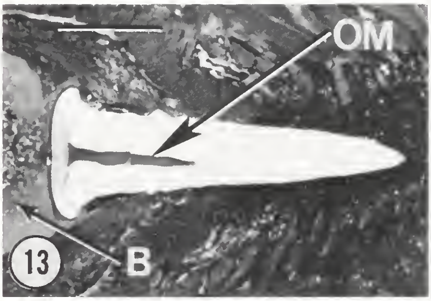


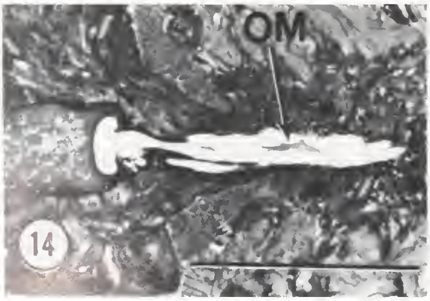












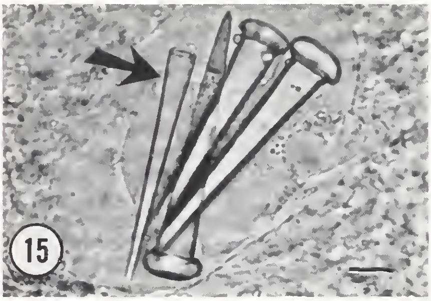


FIGURE 8. Whole mount of a styletocyte of *Paranemertes* sp., showing several reserve stylets and a stylet vacuole (SV). The arrow marks two aberrent stylets that have proximal pieces on their very short shafts. Normally, the stylet shaft develops first, and the proximal piece is not formed until the shaft is well developed. ×500. Scale bar = 50 μm.

FIGURE 9. Whole mount of a reserve stylet sac of *Zygonemertes virescens*. At the onset of stylet formation, a membrane-bound stylet vacuole (SV) forms in the cytoplasm of the styletocyte and becomes filled with a refractile vacuolar matrix, in which a stylet will develop. ×190. Scale bar = 100 μm.

FIGURE 10. Whole mount of two reserve stylet sacs of *Amphiporus formidabilis*. Each sac has a newly formed, conical organic matrix (OM) forming at the edge of the stylet vacuole. ×135. Scale bar = 100 μm.

in the cytoplasm of the styletocyte (Fig. 9). The vacuole is filled with slightly refractile material that is darkly stained in PAS tests. After the vacuole has enlarged, the organic matrix of the stylet shaft begins to develop on the inside of the vacuolar membrane, forming a highly refractile, tapered sliver that is weakly PAS positive (Figs. 10, 11). At an early stage of differentiation, the organic matrix is smooth in most species, but in *Amphiporus bimaculatus* it has fine longitudinal striations (Fig. 12). In fully developed stylets, the organic matrix comprises the core of the shaft and the central part of the proximal piece. The matrix remains visible in proboscides that have been decalcified with E.D.T.A. (6%; pH 7.4), while the rest of the stylet is completely dissolved within several hours. Such decalcified whole mounts as well as reconstructions of serial sections show that the shape of the organic matrix is similar to the general morphology of the cortex (Fig. 13). In *Paranemertes peregrina*, for example, the organic matrix shows helical twists that correspond to the helically twisted grooves and ridges found along the shaft of the stylet (Fig. 14).

After assembly of the organic matrix has begun, the calcified cortex of the shaft becomes deposited around the matrix. The contents of the stylet vacuole diminish as the stylet increases in size (Fig. 15). In well-developed stylets, there is very little vacuolar material left, and the membrane of the stylet vacuole is difficult to detect, except near the junction of the proximal piece and the shaft, as there is a slight indentation in the profile of the stylet in this region (Fig. 16).

The proximal piece is gradually added to a well-developed shaft. Slightly translucent, spherical granules of unknown function are often visible on the shaft in the vicinity of the proximal piece (Fig. 17). As the stylet is being formed in the stylet vacuole, refractile granules that measure about 0.5 to 1.0 μm in diameter can also be seen in the cytoplasm surrounding the stylet vacuoles (Figs. 18, 19). Their abundance varies in the different species examined, and their function is unknown.

In specimens of *Amphiporus formidabilis* and *Emplectonema gracile* monitored over a period of several weeks, stylets were observed to attain full length and have well-developed proximal pieces in 2 to 4 weeks, while in *Tetrastemma* sp. this amount of growth took 3 to 8 weeks (Table I). Additional calcification occurs beyond this point, however, as the overall width of the stylet is increased. The duration of this appositional growth was not determined in this study.

At least one developing stylet was visible in the majority of the specimens examined, regardless of whether the worms had been fed or starved. The rate of

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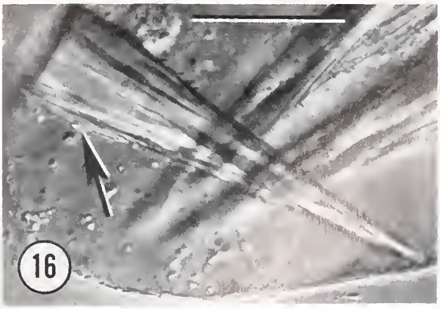
FIGURE 11. Whole mount of a reserve stylet sac of *Zygonemertes virescens* that shows a fairly well-developed organic matrix (OM) in a stylet vacuole. The organic matrix is basophilic in sectioned material and remains visible in whole mounts of the styletocyte that have been decalcified with E.D.T.A. Nomarski Differential Interference Contrast (D.I.C.) optics. ×155. Scale bar = 100 μm.

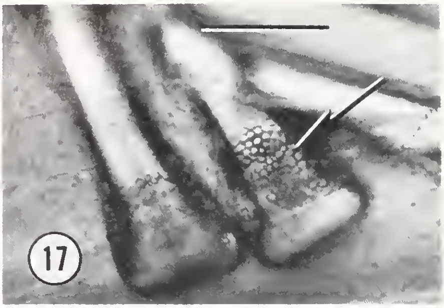
FIGURE 12. Whole mount of a reserve stylet sac of *Amphiporus bimaculatus*, showing a longitudinally striated organic matrix (arrow) within the stylet vacuole. D.I.C. optics. ×155. Scale bar = 100 μm.

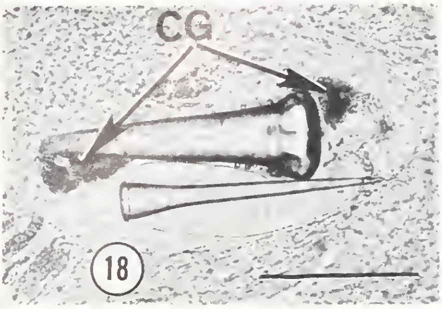
FIGURE 13. Longitudinal section of a central stylet in *Amphiporus formidabilis* attached to its basis (B). The shape of the organic matrix (OM) corresponds to that of the cortex. ×280. Scale bar = 50 μm.

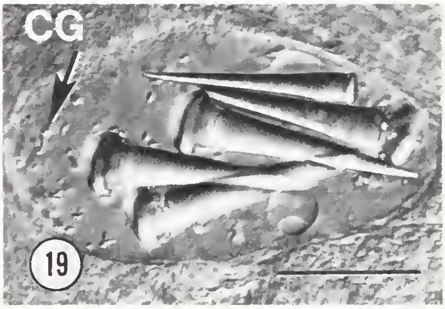
FIGURE 14. Longitudinal section through the central stylet and basis of *Paranemertes peregrina*. Decalcified whole mounts and reconstructions of serial sections reveal that the organic matrix (OM) is helically twisted; these helices correspond to the helical grooves and ridges that occur along the shaft. ×605. Scale bar = 100 μm.

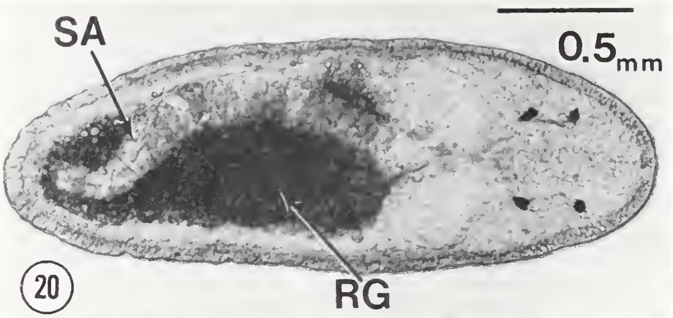
FIGURE 15. Whole mount of a reserve stylet sac of an adult *Tetrastemma* sp., showing a developing stylet with an incompletely formed proximal piece (arrow). As stylets increase in size, the size of the stylet vacuole diminishes. ×590. Scale bar = 10 μm.

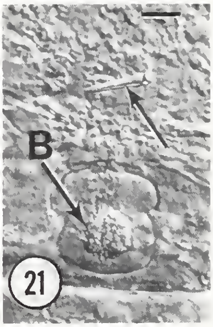


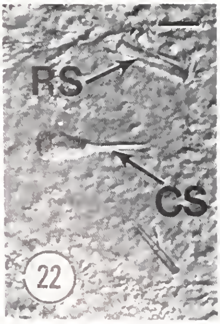


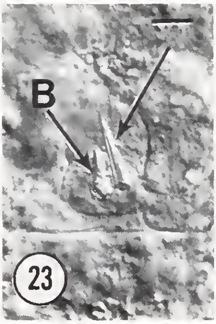












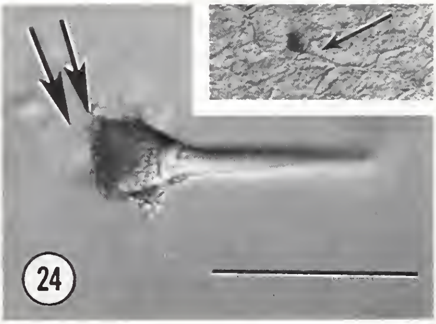


FIGURE 16. Whole mount of a reserve stylet sac of *Emplectonema purpuratum* dissected from the proboscis. The vacuolar membrane is barely discernable near the edge of a well-developed stylet (arrow). This surrounding membrane appears to be left behind in the styletocyte, when the stylet is transferred from the cell to replace the central stylet. D.I.C. optics. ×350. Scale bar = 50 μm.

FIGURE 17. Whole mount of some reserve stylets of *Amphiporus bimaculatus*. Spherical, translucent granules (arrow) of unknown function can be seen at the junction of the proximal piece and shaft in the developing stylets of most species. D.I.C. optics. ×395. Scale bar = 50 μm.

TABLE I

Rates of stylet formation.

Species	Age	Duration of stylet elongation*	Approximate length of mature stylet (μm)
Amphiporus formidabilis	Adult	2–4 weeks	200
Emplectonema gracile	Adult	2–3 weeks	235
	Larva (4 days)	~3 days	15
Tetrastemma sp.	Adult	3–8 weeks	65
	Larva (3 weeks)	4–5 days	12

* Time required for stylet to reach full length and have a proximal piece formed on shaft.

stylet formation did not appear to be significantly different in starved *versus* fed animals. The number of reserve stylet sacs usually remained constant during the several weeks of observation. One small specimen of *Amphiporus formidabilis*, however, increased its number of reserve stylet sacs from 6 to 8 over a period of 2 weeks, while two other specimens lost a sac, presumably during replacement of the central stylet.

Development of the stylet apparatus in larvae

Embryos of *Emplectonema gracile* begin hatching about 1 1/2 days after fertilization, when cultured at 10–12°C, and develop into 200 μm long, free-swimming larvae by the time they are 2 1/2 days old (Table II). Stylets first appear in 4 day old larvae. They can be seen as a pair of small (<5 μm) slivers of refractile material on either side of the developing proboscis. At this stage, there is no sign of the

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FIGURE 18. Whole mount of the cytoplasmic granules (CG) in the styletocyte of *Amphiporus formidabilis*. The origin of these granules and their role in stylet formation remain unclear. ×210. Scale bar = 100 μm.

FIGURE 19. Whole mount of the cytoplasmic granules (CG) in the styletocyte of *Amphiporus imparispinosus*. The abundance of these granules varies considerably among different species. D.I.C. optics. ×395. Scale bar = 50 μm.

FIGURE 20. *Tetrastemma* sp.; whole mount of an approximately 4 week old specimen that had been removed from its egg mass. The proboscis is well formed and contains a stylet apparatus (SA), while the rudimentary gut (RG) consists of an undifferentiated mass of yolk. ×35. Scale bar = 500 μm.

FIGURE 21. *Tetrastemma* sp.; whole mount of the stylet apparatus in an approximately 3 1/2 week old specimen that was in the process of attaching its first stylet (arrow) to the basis (B). Reserve stylets begin to form when the worm is about 3 weeks old, and shortly thereafter, they are moved into the lumen of the proboscis, apparently by muscular contractions. D.I.C. optics. ×545. Scale bar = 10 μm.

FIGURE 22. *Tetrastemma* sp.; whole mount of a newly attached central stylet (CS) in a 3 1/2 week old specimen. The central stylet is derived from a reserve stylet (RS) that is made in the reserve stylet sac and transferred to the basis. D.I.C. optics. ×545. Scale bar = 10 μm.

FIGURE 23. *Tetrastemma* sp.; whole mount of an incorrectly positioned central stylet (arrow), found next to the basis (B) in a 4 week old larva. The transfer of reserve stylets to the basis takes several hours to 2 days in this species, and often several reserve stylets are moved into the lumen of the proboscis during the process. D.I.C. optics. ×545. Scale bar = 10 μm.

FIGURE 24. *Tetrastemma* sp.; whole mount of a central stylet in the proboscidial lumen of an adult (inset), as the stylet was being replaced by a new one. The stylet was removed from the proboscis and found to attach to the microscope slide, by way of the adherent basis granules (double arrows). D.I.C. optics. ×540. Scale bar = 50 μm.

TABLE II

Events in the development of the stylet apparatus in Emplectonema gracile *(10–12°C).*

Day	Event
1½	Hatching begins.
2½	200 μm long, free-swimming larva with no stylet apparatus.
4	Reserve stylets present; no basis or central stylet.
5	Basis begins to form.
6	Basis fully formed, surrounded by basis sheath.
7	Reserve stylet moved to basis.
8	Almost all larvae with basis, central stylet, and reserve stylets.

central stylet or basis. The basis becomes visible in larvae that are 5 days old, and is completely formed and surrounded by a basis sheath in larvae that are about 6 days old.

In 7 day old larvae, reserve stylets are moved from the styletocyte and placed on the basis. The entire process takes only a few hours, and it occurs at nearly the same time in almost all the healthy larvae in the culture. Stylets are squeezed from their position at the sides of the proboscis, apparently by contractions of the proboscidial musculature and/or the contractile elements in the epithelial cells surrounding the styletocyte itself. The stylets come to lie anterior to the basis in the lumen of the proboscis, and are moved about by contractions of the body. After the proximal piece is oriented toward the basis, the stylet is moved posteriorly. Contractions of the longitudinal muscles in the proboscis seem to supply the motive force for this movement, and the stylet becomes attached in a slight depression at the anterior end of the basis.

Embryos of *Tetrastemma* sp. reared at 10–12°C develop a proboscis in the region dorsal to their yolk-filled rudimentary gut, and the first stylets form toward the posterior end of the proboscis, when the worms are about three weeks old (Fig. 20). Within 2 to 3 days following the appearance of the reserve stylets, the basis is formed, and shortly thereafter a reserve stylet is transferred to the basis. The entire process of stylet attachment takes several hours to two days, and it occurs at markedly different times in worms that develop in the same egg mass.

The reserve stylet sac epithelium that surrounds the styletocyte appears to be well developed at the onset of stylet transfer. This epithelium seems to contract during expulsion of the stylets and can remain in a wrinkled form, independent of surrounding muscular contractions. In larvae that are attaching a stylet to the basis, one to several stylets can be seen in the lumen of the proboscis (Fig. 21). These stylets are either moved about by contractions of the body musculature or they remain attached to the inner epithelial lining of the proboscis, apparently because of glandular secretions produced by the proboscidial epithelium. On one occasion, the proboscis was everted under the pressure of the coverslip, and the three reserve stylets in the lumen of the proboscis remained attached to the proboscis, even after the proboscis was turned fully inside out.

Following the transfer of the stylets to the lumen of the proboscis, the basis can be seen to extrude a string of granules in the direction of a stylet. The granules were never observed in the process of attaching to the stylet, but in some cases a small amount of material that might have been derived from these granules could be seen adhering to the proximal piece of stylets in the lumen of the proboscis. The final placement of the stylets on the basis appears to be dependent on muscular contractions that move the stylets toward the basis, and attachment may be aided by granules extruded by the basis (Fig. 22).

Occasionally, a stylet can be seen as it is moved posteriorly to a position behind the correct attachment site on the basis (Fig. 23). It is not clear whether such misplaced stylets can be subsequently maneuvered to their correct position on the basis.

Prey capture and stylet replacement

When amphipods are added to a dish containing adult *Tetrastemma* sp., the worms begin to crawl actively about, and within several minutes most of the nemerteans successfully capture a prey. Prey capture in this species is similar to that described for other suctorial-type feeders (McDermott, 1976). At the onset of attack, the worm rapidly everts its proboscis and coils it around the prey. The central stylet stabs the ventral side of the amphipod several times, and shortly thereafter the prey is immobilized; some prey, however, remain quite active while they are entwined by the proboscis, and on one occasion, an amphipod succeeded in pulling out the proboscis of an attacking nemertean. After the prey is immobilized, the nemertean sucks out the soft tissues of the amphipod and leaves a cleaned exoskeleton behind. The entire feeding episode is usually completed within thirty minutes.

In the thirty feeding encounters that were observed, the loss of a central stylet was seen only twice. In both cases, the central stylet was replaced without the actual attachment of the stylet to the basis being observed. On one occasion, the basis was found lacking a stylet as well as granules in its anterior half, and two stylets were observed in the lumen of the proboscis. In the cytoplasm of one of the styletocytes, there were two membranous structures, each of which comprised the outline of a fully formed stylet. These membranes most likely represented the remains of the stylet vacuoles that were left behind after the stylets had been transferred from the cell.

In addition to this instance of stylet replacement, the central stylet was found to be missing in several specimens of *Amphiporus formidabilis*, *Emplectonema gracile*, and *Tetrastemma* sp. that had been maintained in the laboratory without food. In each case, at least one stylet, and sometimes as many as five stylets, could be seen in the lumen of the proboscis, in front of the basis. The stylets could be seen in the proboscidial lumen of a specimen that was not greatly flattened, and they were sometimes observed up to a centimeter away from the basis; both of these facts tend to refute the possibility that compression of the specimen during examination caused an artifactual dislodging of supernumerary stylets into the lumen.

In one specimen of *Tetrastemma* sp., the stylet in the lumen had numerous refractile granules attached to it. These granules were similar in size and structure to those of the basis, suggesting that the stylet represented the old central stylet that was being replaced. When the coverslip pressure was increased to force the stylet from the proboscis, the stylet became firmly attached to the slide by the adhering granules (Fig. 24). The stylets in the proboscidial lumen of the *A. formidabilis* and *E. gracile* specimens that were observed in the process of replacing their stylets also had granules attached to their proximal pieces. Stylet replacement was not observed to completion in any of these specimens, as movement of the stylet was eventually halted, following the treatment with $MgCl_2$ and the compression of the worm that is required to observe the stylets within the body.

TABLE III

The numbers of reserve stylets in worms freshly collected from the field and worms starved in the laboratory.

Species	Field or starved (length of starvation)	N	Reserve stylet sacs/worm		Reserve stylets/sac		Average no. of stylets/worm
			Avg.	Range	Avg.	Range	
Amphiporus fomidabilis	Field	10	8.8	7–10	1.7	1–3	15.0
	Starved (3 weeks)	6	8.8	8–11	1.9	1–3	16.7
Emplectonema gracile	Field	10	2.0	2–2	6.9	6–10	13.8
	Starved (3 weeks)	8	2.0	2–2	8.9	8–12	17.8
Paranemertes peregrina	Field	10	2.0	1–3	3.2	2–10	6.4
	Starved (8 weeks)	7	2.3	2–3	4.1	2–6	9.4
Tetrastemma sp.	Field	10	2.0	2–2	3.2	1–5	6.4
	Starved (6 weeks)	17	2.0	2–2	3.8	2–5	7.6

Number of reserve stylets in starved vs *fed worms*

To determine whether the total number of stylets differs in starved *vs* fed populations of worms, stylets were counted in specimens freshly collected from the field and in nemerteans that had been starved in the laboratory for up to 8 weeks. In all species examined, the worms that had been starved in the laboratory had a greater number of stylets than those collected from the field (Table III). This difference was not significant at the $P = 0.05$ level in all species tested (Mann-Whitney U, one-tailed test).

Laboratory experiments were also conducted to monitor the number of stylets in starved and fed groups of *Amphiporus formidabilis.* The experimental worms were kept in individual containers with running sea water, and the number of reserve stylets was monitored in slightly compressed whole mounts on a weekly basis. The increase in the number of reserve stylets tended to be greater in starved specimens than in those that were offered prey (Fig. 25). Starved *A. formidabilis* had an average of 2.6 more stylets at the end of 5 weeks, while worms that were fed amphipods *ad libitum* lost an average of 1.3 stylets. This difference in the number of stylets was found to be statistically significant at the $P = 0.05$ level (Mann-Whitney U, one-tailed test).

DISCUSSION

The nine species examined in this study represent three of the six families that comprise the hoplonemertean suborder Monostilifera (classification of Gibson, 1972). The only other nemerteans with stylets belong to the suborder Polystilifera. Polystiliferous hoplonemerteans comprise a small group of pelagic forms and a few benthic species that are distinguished from monostiliferans by having numerous minute stylets on each basis (Brinkmann, 1917). It is not known if the stylets of these hoplonemerteans have the same composition as those found in monostiliferans, or if stylet formation occurs in a similar manner in the two groups.

Monostiliferans of the genus *Gononemertes* have neither reserve stylets nor a central stylet. Coe (1943) has postulated that the loss of the stylet apparatus in these worms is related to their endosymbiotic life style. Members of the genus

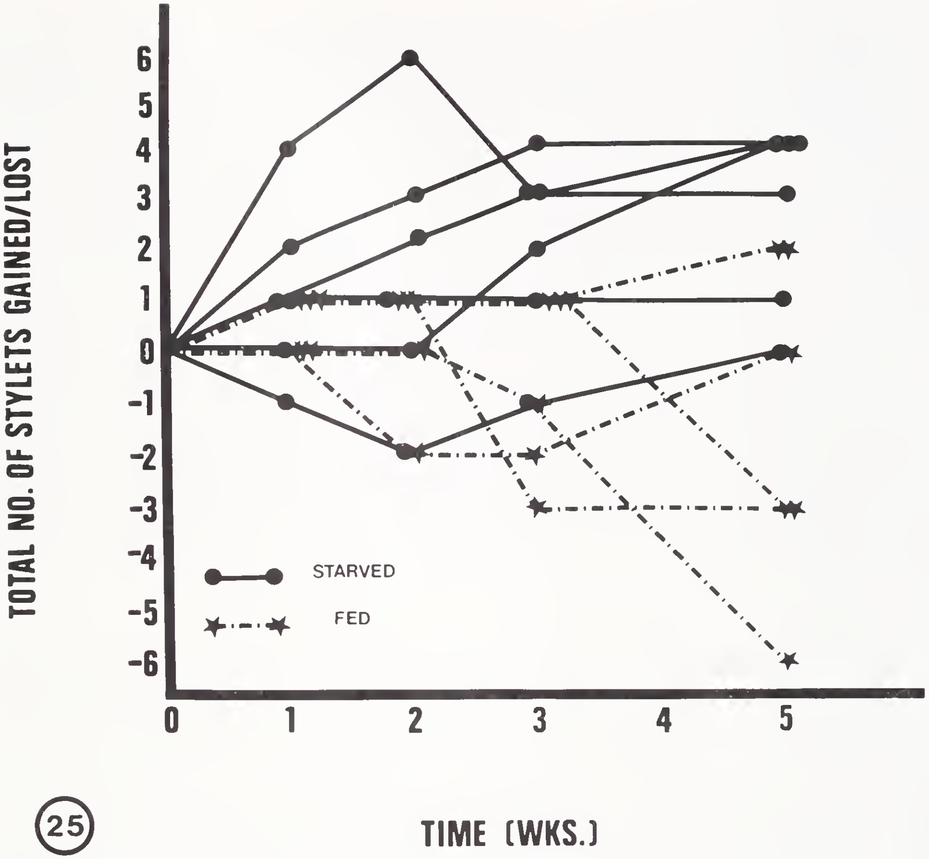

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FIGURE 25. The number of reserve stylets gained/lost in starved *vs* fed specimens of *Amphiporus formidabilis* during a 5 week laboratory experiment. Twelve worms, collected from the same locality, were maintained in individual containers with running sea water. Half the worms were starved, while the other half were fed amphipods *ad libitum*. The number of mature reserve stylets was counted in live whole mounts, on a nearly weekly basis. The starved worms had an average of 2.6 more stylets at the end of the 5 week period, while the worms fed amphipods lost an average of 1.3 stylets (statistically significant difference at the $P = 0.05$ level; Mann-Whitney U, one tailed test). Since both groups of worms were observed to form stylets at approximately the same rate, it was concluded that prey capture causes a significant depletion in the number of reserve stylets.

Carcinonemertes (suborder Monostilifera) live on various crabs and eat the eggs of their hosts (Wickham, 1979). Adult worms in this genus possess a central stylet and basis, but they lack reserve stylet sacs (Coe, 1902). Newly hatched larvae of *C. epialti* do not have a central stylet, and an ultrastructural study of the nascent larva failed to reveal any styletogenic cells (Stricker and Reed, 1981). Coe (1943) has suggested that a temporary reserve stylet sac develops and produces the adult central stylet, but further investigations are needed.

Aside from these two aberrent genera, stylet formation in monostiliferous hoplonemerteans occurs in a fairly uniform manner. The following discussion applies to the nine species examined in this study and compares some of the basic features of stylet formation displayed by nemerteans in general.

Site of stylet formation

Although most authors (*e.g.*, Böhmig, 1929; Hyman, 1951; Gibson, 1972) have agreed with the view first presented by Bürger (1895) that stylet formation takes place intracellularly in the reserve stylet sacs, a few have claimed that stylets are formed next to the basis (Montgomery 1894; Coe, 1904, 1905). Montgomery (1894) maintained that the morphologies of the central and reserve stylets are markedly different. Other authors, however, have found the structure of the central and reserve stylets to be identical (Bürger, 1895; Coe, 1904, 1905; Stricker and Cloney, 1981). Apparently, Montogmery did not know that reserve stylets occur within intracellular vacuoles. His drawing of a reserve stylet (Montgomery, 1894) seems to have included the vacuolar matrix in addition to the stylet itself, which may account for his misconception regarding the structure of the two kinds of stylets.

Our observations that the first stylets in larvae develop intracellularly within the prospective reserve stylet sacs, before the basis or central stylet develop, agree with those of Iwata (1960). In the adults of all nine species we examined, stylets were always observed to develop in the styletocyte, and styletogenic cells were not seen at any location other than in reserve stylet sacs, confirming the view proposed by Bürger (1895) that stylet formation in nemerteans occurs intracellularly within the reserve stylet sacs.

Rates of stylet formation

In the adult nemerteans we examined, stylets reached full length and had well-developed proximal pieces in two to eight weeks. It should be emphasized that stylets are not fully formed when they reach this stage. Appositional growth continues for an undetermined length of time, until the stylet attains the width of a central stylet. Thus, the times reported for stylet formation in this study underestimate the minimum time it takes for a stylet to become fully formed. In any case, our observations of stylet formation indicate that styletogenesis in the species we examined takes substantially longer than in other species studied. Sunberg (1979), for example, has shown that in *Tetrastemma laminariae* there is a statistically significant greater number of stylets in specimens that have been starved for 8 days than in those freshly collected from the field, indicating that the assembly of reserve stylets can be accomplished in less than 8 days. According to Bartsch (1973), *Tetrastemma melanocephalum* can regenerate its proboscis within 2 weeks, which implies that stylets can also be formed in this amount of time.

The fact that most of the nemerteans examined in this study contained at least one developing stylet supports Coe's (1943) contention that stylet formation occurs more or less continuously in nemerteans. Reisinger (1926) reported that stylet formation occurs only in specimens that had been fed prey, but we observed developing stylets in worms that had been fed prey as well as in starved specimens.

Although it is probable that stylet formation occurs throughout the life of the worm, whether or not it is fed, there may be an upper limit as to the number of stylets a worm can form. Such a maximum output would account for the fact that most of the specimens that failed to form stylets during the course of this study seemed to be older worms, judging from the size of their bodies and stylets.

Stylet replacement

In nearly all hoplonemerteans, the first stylet apparatus formed is much smaller than that found in the adult. Therefore, the central stylet must be replaced as the worm increases in size. Coe (1943) has observed a specimen of *Emplectonema gracile* that had a small stylet with an attached basis, apparently in the process of being replaced by a larger stylet and basis. Corrêa (1949, 1954) has made similar observations on other species. According to Gibson (1972), the larvae of some species, in which the adults are quite small (*e.g.*, *Prostoma* and *Tetrastemma*), form a stylet apparatus that can be as large as that found in the adult. These species would not need to replace their stylet except following its loss. The first stylets formed by the *Tetrastemma* sp. that we examined were considerably smaller than the fully formed stylets found in adults; thus, even in this species, the first stylets must be replaced by larger ones during growth of the animal. The only other explanation to account for this increase in stylet size is that further calcification occurs while the stylet is on the basis, but there is no evidence to support this view.

Stylet replacement is also required following loss or damage to the stylet, as occurs during prey attack. According to Reisinger (1926), specimens of *Prostoma rubrum* that were fed oligochaete prey lost their stylet after nearly every feeding episode. In *Paranemertes peregrina*, on the other hand, stylet replacement was not observed in over one hundred specimens examined (Roe, 1970; Stricker and Cloney, 1981). In this study, specimens of *Tetrastemma* sp. that were observed directly following prey attack were only rarely seen to replace their stylets. Starved worms in all four species examined, however, tended to have more reserve stylets than did those that were freshly collected from the field. Since the rates of stylet formation were not seen to differ in the two groups, we conclude that stylet replacement occurs frequently enough in the field following prey attack to cause a depletion in the number of reserve stylets. Although observations of *Tetrastemma* sp. in the laboratory failed to reveal a high frequency of stylet replacement, it is possible that stylets are replaced more frequently in the field, where a greater variety of prey is encountered.

Worms that had been starved for several weeks were also observed in the process of replacing their stylets. In most cases, the stylet that was being replaced was significantly smaller than the new stylet, suggesting that the stylet apparatus was still in the process of growing. On several occasions, the new and old stylets were of equal size, which supports the view held by Reisinger (1926) that stylet replacement occurs throughout the life of the animal, even after growth of the stylet apparatus has ceased. During stylet replacement several stylets were often observed in the proboscidial lumen. Reisinger (1926) also reported that in *Prostoma rubrum*, the entire complement of reserve stylets may be transferred into the lumen during stylet replacement. These observations, coupled with the fact that replacement of the stylet is required at least occasionally following prey attack as well as during times when the animal is not feeding, may account for the relatively large numbers of reserve stylets produced by nemerteans.

The complete sequence of stylet replacement in adult worms was not observed in this study, as the process is apparently terminated by the flattening of the specimen, or the treatment with $MgCl_2$, that is required to observe stylets *in situ*. Reisinger (1926) encountered similar difficulties in observing stylet replacement in *Prostoma rubrum*. The several stages of replacement that we observed indicate that the process occurs in larvae, as well as in adults, in the following manner. One to several stylets are transferred from the styletocyte into the duct of the reserve stylet sac, apparently by contractions of the surrounding muscles and/or the epithelial cells that envelop the styletocyte. This transfer may involve either a partial or complete loss of the contents of a styletocyte, judging from the apparent remains of stylet vacuoles in the styletocyte and the disappearance of an entire stylet sac following stylet transfer. As shown in *Paranemertes peregrina* (Stricker and Cloney, 1981), the duct of a reserve stylet sac communicates with the proboscidial lumen near the central stylet, so that once the reserve stylets are forced through the duct, they will reach the vicinity of the basis. Stylets in this region of the proboscis are moved about by muscular contractions, and thus oriented so they can be attached to the basis. The ultrastructure of the proximal piece differs from that of the shaft (Stricker, unpublished observations); this difference may increase the chances of the proximal piece being moved toward the basis by muscular contractions. Alternatively, granules may be extruded from the basis and become attached more readily to the proximal piece than the shaft, thus facilitating the correct transfer of the stylet to the basis. The final attachment to the basis appears to be dependent upon the adhesive properties of the basis granules. The ultrastructure of the basis granules in *P. peregrina* suggests that they are adhesive (Stricker and Cloney, 1981), and the fact that an isolated portion of the basis was found to adhere to a microscope slide also supports this view.

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SOME PROPERTIES OF AN AGGLUTININ IN THE HAEMOLYMPH OF THE POND SNAIL *LYMNAEA STAGNALIS*

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ABSTRACT

A broad spectrum agglutinin in the haemolymph of the pond snail, *Lymnaea stagnalis*, is described. At dilutions ranging up to 1/2048 it agglutinated erythrocytes of several sources, bacteria and yeast cells. Haemolymph of some specimens agglutinated all types of cells, whereas that of others did not agglutinate human and sheep erythrocytes.

In inhibition tests with a variety of carbohydrates, D-galactose, L-forms of some monosaccharides, and 3 polysaccharides, especially *L. stagnalis* galactogen, were good inhibitors.

The agglutinin is a protein with a M.W. of approximately 60,000.

It was demonstrated *in vitro* that the agglutinin probably has opsonizing properties.

INTRODUCTION

Agglutinins occur in the haemolymph of gastropod molluscs and of other invertebrates (*e.g.* Pauley *et al.*, 1971; Acton and Weinheimer, 1975; Tripp, 1975; Stein and Basch, 1979). Such haemolymph agglutinins may play a role in internal defense as opsonins (Renwrantz *et al.*, 1981).

In *Lymnaea stagnalis* the haemolymph has opsonic activity (Sminia *et al.*, 1979). Also, since clotting of foreign particles was observed in the circulation of *L. stagnalis* (Van der Knaap *et al.*, 1981a), we investigated the haemolymph for the presence of agglutinins. These were found and some of their properties are described here.

MATERIALS AND METHODS

Agglutinin assays

Serum was prepared from haemolymph (Van der Knaap *et al.*, 1981a), collected from laboratory bred mature specimens (shell height 27–32 mm) of the pond snail, *Lymnaea stagnalis*. Titrations were performed in Cooke microtiter plates with V-bottomed wells of 200 μl capacity. Serum was serially two-fold diluted with phosphate-buffered saline (PBS; 0.01 *M* Phosphate, 0.15 *M* NaCl, pH 7.4) in 100 μl quantities. To each well, 100 μl of a 1% (v/v) suspension of foreign cells in PBS was added. For undiluted serum, the osmolality was adjusted to that of human serum, and 5 μl of a 10% cell suspension was added. After incubation for 2 h at room temperature titers were read macroscopically as the reciprocals of the greatest

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dilutions giving total agglutination. Foreign cells used were human (bloodgroups A1, A2, B, AB and O), sheep and rabbit red blood cells, *Staphylococcus saprophyticus* and *Escherichia coli* (bacteria), and *Saccharomyces cerevisiae* (yeast). The erythrocytes were provided by the Department of Haematology, University Hospital, Vrije Universiteit, Amsterdam; the bacteria were grown overnight in Oxoid nutrient broth, harvested and washed in PBS; the yeast was purchased from a local bakery.

Adsorption tests

After the above titrations, 150 μl of supernatant fluid was carefully transferred from each well into a new well and 10 μl of a 10% cell suspension was added to each well; these cells were either of the same type as in the original test, or of another type.

Induction of agglutinin

Since agglutinin titers varied considerably among individual snails (see results), 3 experiments were performed to ascertain whether very high titers (in type I snails, see results) had been induced by previous or persistent contact between the defense system and foreign materials. In the first experiment, agglutinin titers were determined in haemolymph samples of 50 field collected specimens of *L. stagnalis*. In the second experiment, groups of 10 snails with very low agglutinin titers were injected (Van der Knaap *et al.*, 1981a) 3 times at 1 wk intervals with the following substances: 10 μl of snail Ringer (Van der Knaap *et al.*, 1981a; control snails), 10 μl of total haemolymph from snails with very high agglutinin titers, or 10 μl of snail Ringer containing 1×10^8 live bacteria (grown as described above). Bacteria used were *E. coli, S. saprophyticus*, and 4 unidentified species. The latter were isolated by spreading 100 μl quantities of snail haemolymph on nutrient broth (Oxoid) agar plates, which were then incubated at 37°C. Of the colonies which had grown overnight, 4 were selected which could be distinguished by their shape and color, and which arose from haemolymph of snails with very high agglutinin titers only. One wk after the third injection, haemolymph was sampled and agglutinin titers of individual samples were determined. It was established whether titers in experimental animals differed from those in controls. In the third experiment, 7 snails with very high agglutinin titers were kept isolated for 2 wks; then, freshly laid egg masses were collected and pooled. When the shell height of the snails hatched from these egg masses reached 27 mm, agglutinin titers were determined in haemolymph samples of 200 randomly chosen specimens.

Agglutination inhibition

In inhibition tests (and all other tests to be mentioned hereafter) only human A erythrocytes were used as foreign cells. A group of saccharides (see Table II) based on comparable published results (*e.g.* Khalap *et al.*, 1970), was tested for their capacity to inhibit agglutination. Of monosaccharides, both D- and L-isomers were tested. Saccharides were purchased from Sigma or Merck; galactogen, prepared from *L. stagnalis* albumen glands, was a gift from Prof. Dr. J. Joosse. Qualitative tests were performed by making dilution series of snail serum in PBS (see above) and adding 50 μl of PBS containing 3.6 mg of a saccharide to each well. After incubation for 1 h, 50 μl of a 2% erythrocyte suspension was added to each well, and titers were read 2 h later. Quantitative tests were performed by making a two-fold dilution series of each saccharide (resulting in 50 μl quantities of PBS containing 7.2, 3.6, 1.8, 0.9 and 0.45 mg sugar per well) and adding 100 μl of a fixed concentration of snail serum to each well; this serum concentration contained 4 times the minimum concentration required for total agglutination in the absence of an inhibitor. After incubation for 1 h, and adding 50 μl of a 2% erythrocyte suspension, titers were read as above.

Nature of agglutinin

To test for stability of agglutinin to temperature, batches of pooled serum (from 10 snails) were heated to 40, 60, 80 and 100°C for 30 min. Also, pooled serum was stored at room temperature for 24 and 48 h. Sediments, if formed during treatment, were removed by centrifugation, and agglutination titers of the supernatant fluids were determined.

To determine the molecular nature of the agglutinin, ammonium sulphate, trichloroacetic acid (TCA), or ethanol were added to aliquots of pooled serum to final concentrations of 0, 10, 20, 30, 40 and 50% (w/v, w/v, v/v, respectively). Precipitates were removed by centrifugation and the supernatant fluids were dialyzed against 0.9% NaCl (lyophilized and dissolved in the original volume of distilled water in the case of ethanol precipitation) and agglutinin titers were determined.

Enzymatic digestion was done with trypsin (Worthington), pronase E (Merck), Lipase (Sigma), β-glucosidase (BDH), and α-amylase (Boehringer), dissolved in PBS and added to serum in final concentrations of 0.2%. To control serum, heat-inactivated enzymes or PBS were added. After incubation at 37°C for 1 h, agglutinin titrations were performed at 4°C.

Isolation of agglutinin

Since the oxygen-binding blood pigment haemocyanin binds to foreign materials (Van der Knaap *et al.*, 1981b) and might be an agglutinin, it was removed from serum (pH adjusted to 7.2) by ultracentrifugation for 1 h at $100{,}000 \times g$. The haemocyanin pellet was resuspended in the original volume of PBS. Agglutinin titers were determined in the supernatant fluid, in the haemocyanin suspension, and in the original serum.

Based on the results of the above mentioned experiment, additional experiments were performed to isolate agglutinin from haemocyanin-free snail serum by column chromatography. Gel filtration (Ishiyama *et al.*, 1973) on Sephadex G200 (Pharmacia), anion exchange chromatography on Cellex D (Bio Rad) and Bio-Gel A (Bio Rad), and cation exchange chromatography on SP-Sephadex C50 (Pharmacia) were tried. None of the eluted fractions had agglutinating capacity, probably because the agglutinin bound very strongly to the gel matrices (all carbohydrates). Therefore, we chose to perform gel filtration on a non-carbohydrate gel material. Of haemocyanin-free snail serum, 2 ml was eluted from a 90 × 0.9 cm Bio Gel P60 (polyacrylamide, Bio Rad) column with 50 m*M* PBS (flowrate of 1.5 ml/h). Protein-containing peaks (as determined by UV absorption) were lyophilized, dissolved in $^1/_3$ of the original volume of distilled water, and tested for agglutinating activity.

Function of agglutinin

To assess whether agglutinin has opsonizing properties, *in vitro* phagocytosis of formalized human erythrocytes by amoebocytes was measured in haemolymph of snails with high agglutinin titers (see results, *agglutinin assays*) and in that of snails with low titers (method: Sminia *et al.*, 1979).

RESULTS

Agglutinin assays

Agglutinating activity was detected in the haemolymph of all *L. stagnalis* specimens tested. Among individual snails titers varied considerably (0–2048, see Table I). Variation occurred according to a fixed pattern and the snails could be divided into 2 groups: those whose serum agglutinated all types of cells tested (type I snails), and those whose serum did not agglutinate human and sheep erythrocytes, but did agglutinate rabbit erythrocytes, bacteria and yeast (type II snails). Of 200 laboratory-bred snails, 184 were of type II; the remaining 16 were of type I.

Adsorption tests

During the usual titer tests the agglutinating activity of sera was reduced, presumably by adsorption to the foreign cells (final concentration 0.5% v/v). After tests with type I serum agglutination occurred in the supernatant fluids of only the first and very slightly the second well if the same type of cell was used as in the first test. If cells of another type than in the first test were used in the second test, the number of wells still containing agglutinating activity was higher; agglutinin could still be detected in the supernatant fluids of wells 1 and 2 if the titer was very high in the first test (*e.g.* 2048 with rabbit erythrocytes), and of wells 1 through 4 if the titer was low in the first test (*e.g.* 32 with *S. saprophyticus* bacteria). Of far less influence than the titer in the first test was the extent of similarity between the type of cell used in the first and the second test (*e.g.* after testing with human A1 erythrocytes, the supernatant fluid of well 1 agglutinated human A1 or A2 erythrocytes, the fluids of wells 1 and 2 agglutinated human O erythrocytes, whereas those of wells 1, 2 and 3 agglutinated *E. coli* bacteria).

After tests with type II serum, comparable results were found as with type I serum. Although human and sheep erythrocytes were not agglutinated in the first tests (see Table I), they reduced agglutinating activity of type II serum (this could

TABLE I

Agglutinin titers in L. Stagnalis *serum.*

Foreign cell tested	Titer in type I snails	Titer in type II snails
human A1 erythrocytes	256–1024	0
human A2 erythrocytes	128–512	0
human B erythrocytes	128–512	0
human AB erythrocytes	128–512	0
human O erythrocytes	256–1024	0
sheep erythrocytes	128–512	0
rabbit erythrocytes	1024–2048	4–8
S. saprophyticus	16–64	8–32
E. coli	256–1024	256–1024
Sacch. cervisiae	256–1024	256–1024

Highest and lowest titers (reciprocals of the greatest dilutions giving total agglutination) found for 16 type I snails and 184 type II snails are given.

be measured with rabbit erythrocytes, the bacteria, and yeast cells only, see Table I) to the same extent as they did in type I serum.

Induction of agglutinin

Of 50 field collected snails, 49 were type II and 1 was type I. In the haemolymph of all 50 specimens unidentified parasites and bacteria were observed with phase-contrast microscopy. It therefore is unlikely that the higher agglutinin titers in type I snails were induced non-specifically by persistent contact between the defense system and these foreign organisms.

Agglutinin titers in type II snails were unaltered after repeated injections with any of 6 bacterial species (4 of which had been isolated from type I snails), or with type I haemolymph. This makes it highly improbable that certain bacteria or viruses would have specifically induced higher agglutinin titers in type I snails.

Of 200 specimens reared from egg masses laid by type I snails, 120 were type I, whereas in the normal laboratory snail stock only 16 out of 200 were type I. Therefore, it is likely that heredity rather than induction determines whether a snail is of type I.

Agglutination inhibition

Agglutination of human A erythrocytes in type I serum was inhibited by carbohydrates. Since the inhibitory effect of each saccharide varied per experiment, ranges of effectivity observed in 4 qualitative and 4 quantitative tests are listed in Table II.

Nature of agglutinin

Type I serum, heated to 40°C for 30 min, or stored at room temperature for 24 h agglutinated human A erythrocytes with the same titers as fresh serum. If

TABLE II

Inhibition of L. stagnalis *agglutinin by saccharides.*

Monosaccharides	D-Isomer	L-Isomer	Other saccharides	
arabinose	±	±	lactose	±
fructose	0	N.D.	trehalose	±
fucose	0	+	galactosamine	0
galactose	+	+	N-ac-galactosamine	±
glucose	±	++	N-ac-glucosamine	±
mannose	±	++	cellulose	+
rhamnose	N.D.	+	galactogen	+++
ribose	0	N.D.	glycogen	++
xylose	±	±	starch	0

Pooled results of 4 qualitative and 4 quantitative experiments. The range of the inhibitory effects which each sugar (final concentration 18 mg/ml) had in the 4 qualitative experiments is expressed as: 0, inhibition was not observed; ±, titers not reduced–reduced 2 fold; +, titers reduced 2–4 fold; ++, titers reduced 4–8 fold; +++, titers reduced 8–16 fold (*e.g.*, if in the 4 experiments titers without addition of an inhibitor were 512, 512, 256 and 512, and after addition of an inhibitor titers were 256, 128, 128 and 256 respectively, this sugar reduced titers 2, 4, 2 and 2 fold, and is therefore listed as + for 2–4 fold reduction in titer). The same signs are used to indicate how the inhibiting effects of the sugars ranged in 4 quantitative experiments: 0, inhibition was not observed; ±, no inhibition–inhibition with 36 or 18 mg/ml sugar concentration; +, inhibition with 9–18 mg/ml sugar concentration, ++, ditto, 4.5–9 mg/ml; +++, ditto, 2.25–4.5 mg/ml (*e.g.*, if a sugar worked inhibitory at concentrations 18, 9, 9 and 18 mg/ml in the 4 experiments, it is listed as +. N.D.: not done.

TABLE III

Agglutinin titers of serum after addition of ammonium sulphate (in % w/v final concentration), TCA (% w/v), or ethanol (% v/v).

	% final concentration					
Precipitant	0	10	20	30	40	50
Amm. sulphate	1024	1024	1024	512	512	0
TCA	1024	0	0	0	0	0
Ethanol	1024	1024	512	2	0	0

stored at room temperature for 48 h, titers decreased from 1024 to 64. Serum heated to 60, 80 or 100°C for 30 min lost agglutinating activity completely.

The addition of ammonium sulphate, TCA or ethanol to type I serum resulted in a marked precipitation of material, and supernatant fluids lost agglutinating capacity (Table III).

The above results indicate that the agglutinin is proteinaceous. This is corroborated by enzymatic digestion. Control sera (incubated with PBS or heat-inactivated enzymes) agglutinated with titers of 512, whereas sera treated with trypsin or pronase had no detectable agglutinating activity. Lipase, β-glucosidase and α-amylase did not affect agglutinin titers.

Isolation of agglutinin

The agglutinin titer of normal haemolymph was 1024; after removal of haemocyanin the supernatant fluid had a titer of 512 and the resuspended haemocyanin had a titer of 4.

With gel filtration on Bio-Gel P60 most of the agglutinating activity was found in a conspicuous shoulder at the end of the void volume; a small peak eluted later also contained some agglutinating activity. Two other peaks of lower molecular weight materials, but without agglutinating activity, were eluted.

Function of agglutinin

The opsonizing activity of haemolymph (Sminia *et al.*, 1979) is dependent on its agglutinating activity: both the percentage of phagocytosing amoebocytes and the number of ingested foreign cells per amoebocyte reached maximum values within 30 min in type I haemolymph, whereas in type II haemolymph a 45 min exposure was required before these maxima were reached (see Fig. 1).

DISCUSSION

In the present study the suggestion (Van der Knaap *et al.*, 1981a) that heamolymph of *L. stagnalis* would contain agglutinin was confirmed. With human A erythrocytes, serum of a minority of snails (type I) appeared to have very high titers, whereas serum of the majority of snails (type II) seemed to lack agglutinin. After testing a panel of foreign cells it became evident that type II snails do not lack agglutinin, but have a substance which agglutinates only micro-organisms and rabbit red blood cells. Although this substance does not agglutinate human or sheep erythrocytes, the adsorption tests showed that it does bind to these foreign cells. This is a property which it has in common with type I agglutinin. Possibly type I and type II agglutinin are modifications of one molecule, the latter lacking one

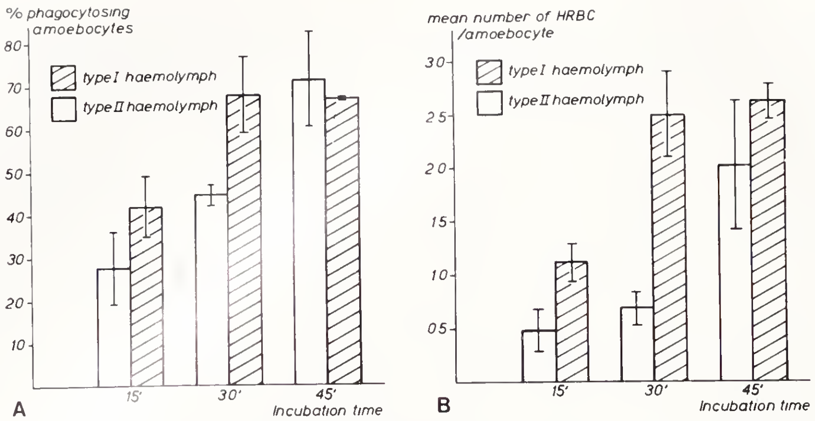

FIGURE 1. *In vitro* phagocytic activity of amoebocytes in pooled haemolymph of 5 type I snails (dashed columns) and of 5 type II snails (open columns). Phagocytosis was stopped after 15, 30 and 45 min, and the percentages of amoebocytes (n = 300) that had phagocytosed (Fig. 1A) and the mean numbers of ingested human erythrocytes per amoebocyte (Fig. 1B) were determined. Means ± S.D. of 3 parallel experiments are given.

or more receptor sites (so that sufficient cross-linking between erythrocytes can not occur). This supposition is in agreement with the observation that, besides haemocyanin, only one substance in pooled snail haemolymph binds to foreign cells (Van der Knaap *et al.*, 1981b).

Differences in agglutinin titers among haemolymph samples of different species within one molluscan genus (Gilbertson and Etges, 1967; Michelson and Dubois, 1977), of different strains within one species (Gilbertson and Etges, 1967; Stanislawski *et al.*, 1976; Michelson and Dubois, 1977), and of different specimens within one strain (Michelson and Dubois, 1977) appear to be a general phenomenon. It has been suggested that it could be of use for taxonomic purposes (Gilbertson and Etges, 1967; Michelson and Dubois, 1977). Repeated injections of type II snails with type I total haemolymph, or with bacteria which could be isolated from type I snails only, did not result in higher titers. Therefore it is unlikely that the stronger agglutinin in type I snails was induced by a viral or bacterial infection. A snail's agglutinin type (I or II) is apparently genetically determined, since 60% of the progeny of type I snails that had been isolated for several weeks were of type I, whereas in the normal laboratory stock this percentage was 8.

In the present study rabbit erythrocytes were the red cells that were agglutinated best. Some other molluscan sera show a similar activity in this respect (*e.g.* Tripp, 1966; Gilbertson and Etges, 1967; Jenkin and Rowley, 1970). Sera of 6 specimens of *Viviparus malleatus* agglutinated rabbit red cells only, and gave titers comparable to those found in type II haemolymph in the present study (Cheng and Sanders, 1962). Possibly in this species differences in agglutinating activity comparable to those in *L. stagnalis* occur among individual snails.

The agglutinins in *L. stagnalis* work non-specifically, since they have a broad action spectrum: type II serum agglutinates at least 3 species of micro-organisms plus rabbit red cells, whereas type I agglutinin is in addition directed against sheep red cells and human erythrocytes, without showing ABO bloodgroup specificity. Another argument for the non-specificity of *L. stagnalis* agglutinin is that adsorption of serum with any of the foreign cells markedly reduced titers against all other cell types. In addition, the non-specificity of *L. stagnalis* agglutinin was evidenced by the inhibition tests: of 24 sugars tested, 9 inhibited the agglutinin in all experiments.

Of 8 D-isomers of monosaccharides, only D-galactose was a good inhibitor: although giving only slight inhibition, it was effective in all experiments. Interaction between D-galactose and molluscan agglutinins is not unusual (Khalap *et al.*, 1970; Baldo *et al.*, 1977). On the other hand, of 7 L-isomers, 5 were good inhibitors. It is tempting to state that these biologically uncommon sugars would be receptor sites par excellence for recognition factors. Strikingly, N-ac-galactosamine and related sugars, which are strong inhibitors of molluscan agglutinins (*e.g.* McDade and Tripp, 1967; Kühnemund and Köhler, 1969; Khalap *et al.*, 1970; Arimoto and Tripp, 1977; Michelson and Dubois, 1977; Uhlenbruck *et al.*, 1979) had little or no effect on *L. stagnalis* agglutinin. Polysaccharides seem to be stronger inhibitors than the monomers that they are composed of: cellulose and glycogen were good inhibitors, whereas D-glucose was not; obviously the nature of the linkage between the monosaccharides is essential, since starch had no inhibiting effects. Poly-D-galactose, *i.e. L. stagnalis* galactogen, proved to be by far the best inhibitor in the present study. This is not surprising, since galactogens interact strongly with molluscan agglutinins (Uhlenbruck *et al.*, 1979).

The agglutinin is obviously of proteinaceous nature because it was precipitated by ammonium sulphate, ethanol, and TCA; it was unstable to heat and was inactivated by pronase and by trypsin. Since it was not affected by enzymes which degrade lipids and carbohydrates the molecule may be pure protein, or it may contain carbohydrate and lipid moieties which are either masked or not essential for the functioning of the molecule. The agglutinin is not identical with the blood pigment haemocyanin. This was corroborated by the results of gel filtration on Bio-Gel P60 columns. Since protein with agglutinating power was eluted in a shoulder of the void volume, the M.W. must be just less than 60,000. The small peak in the lower M.W. range with agglutinating activity may have contained agglutinin subunits.

Molluscan agglutinins have been shown to act as opsonins, both *in vitro* (*e.g.* Arimoto and Tripp, 1977) and *in vivo* (*e.g.* Renwrantz *et al.*, 1981). Opsonizing activity was demonstrated in the serum of *L. stagnalis* by Sminia *et al.* (1979), and indications were found (Van der Knaap *et al.*, 1981b) that this opsonin is identical with the agglutinin described in the present study. The present study is in agreement with the concept that *L. stagnalis* agglutinin has opsonizing properties: in *in vitro* phagocytosis both the percentage of amoebocytes phagocytosing human erythrocytes, and the number of red cells engulfed per amoebocyte were higher in the stronger agglutinating type I, than in type II haemolymph.

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ELECTRICAL ACTIVITY IN RESPONSE TO LIGHT OF THE OCELLUS OF THE HYDROMEDUSAN, *SARSIA TUBULOSA*

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ABSTRACT

The electroretinogram (ERG) recorded from the ocellus of *Sarsia tubulosa* has a characteristic positive potential change at the onset of illumination, followed by a slower biphasic pulse and a positive deflection at the cessation of illumination succeeded by high-frequency pulses. The amplitude of initial pulse is graded with respect to changes in intensity and wavelength of the light stimulus. The maximum spectral sensitivity lies around 540 nm. Responses to light with opposite polarity were recorded from the optic ganglion which surrounds the ocellus. Differences in response patterns inside and outside the receptive field of the ocellus were mapped. Morphological structures which could give rise to component responses of the ERG and its neuronal pathway are discussed.

INTRODUCTION

Ocelli associated with tentacular structures are common in coelenterates. The number of such ocelli varies from four [*e.g. Sarsia tubulosa* (Sars, 1835)] to sometimes several hundred [*e.g. Spirocodon saltatrix* (Tilesius, 1818)]. The structural complexity of ocelli varies widely from simple epithelium not elaborated into a cup, but with photoreceptor cells (Singla, 1974), to a highly organized eyecup with a lens or a lens-like structure (Weber, 1981a, b). Several types of structures between these two extremes have been reviewed by Yoshida (1973) and Singla (1974).

The eyecup (retina) in *Sarsia* (Fig. 1; Singla and Weber, in preparation) is composed of pigment and photoreceptor cells in almost a 1:2 ratio. The photoreceptors are bipolar and grouped together. Bundles of 2 to 10 long (up to 50 μm in length) distal receptor processes project their apical cilia into the lumen of the ocellar cavity (about 90 μm deep and 50–60 μm wide). The plasma membrane covering the cilium forms lateral microvilli. The cell bodies of photoreceptors lie behind the pigmented cup, 20 to 60 μm from the ocellar cavity. The proximal region of photoreceptors extends as an axon. Synapses occur between neighboring cell bodies and axons of the receptor cells at the optic ganglion which surrounds the ocellus (Mackie, 1971). Second order neurons, as described in other hydromedusae (Toh, *et al.*, 1979; Yamamoto and Yoshida, 1980; Singla and Weber, 1982) have not been observed. Instead, the proximal axons of the bipolar receptor cells group together to form a pair of optic nerves (Singla and Weber, in preparation) which travel around the tentacle base on either side and enter the tentacular ganglion (Mackie, 1971), a swelling of the outer nerve ring.

While most invertebrates have photoreceptors of the rhabdomeric type, all the photoreceptors of jellyfish that have been examined so far are of the ciliary type (Eakin and Westfall, 1962; Eakin, 1963). According to Eakin's theory (1963, 1968,

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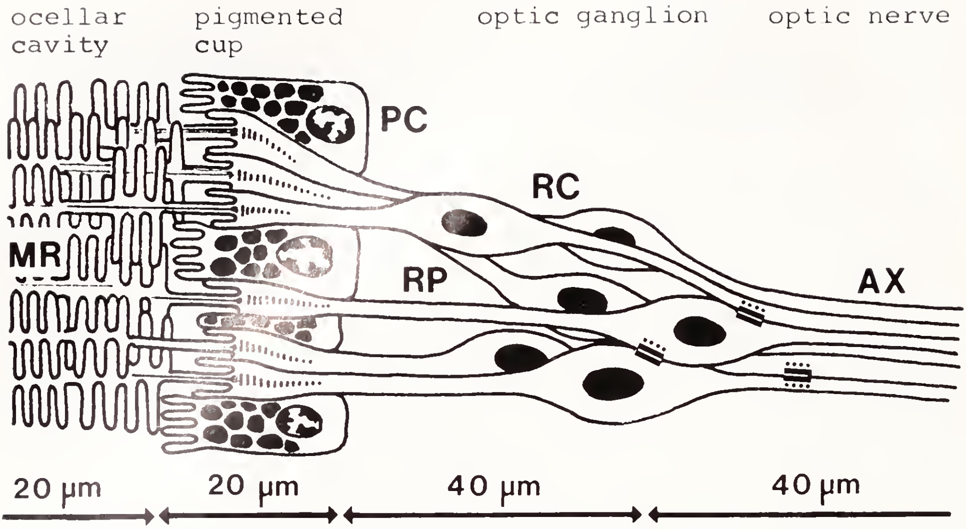

FIGURE 1. Summary diagram of receptor cell arrangement in the ocellus of *Sarsia tubulosa*. AX, axon; MR, microvilli region; PC, pigment cell; RC, receptor cell; RP, receptor cell process.

1979) of the two lines of evolution of photoreceptors, the photoreceptor cells of Cnidaria belong to the same evolutionary line as those of vertebrates. While the rhabdomeric photoreceptors respond with a depolarization, cilia-derived photoreceptors are hyperpolarizing in general. The question then arises whether the physiology of *Sarsia's* receptors is also like that of vertebrate receptors?

In this paper I describe the electroretinogram of a jellyfish, *Sarsia tubulosa* (Hydromedusae), and map the different patterns of photo-induced responses at the optic ganglion which surrounds the receptive field of the ocellus.

MATERIALS AND METHODS

Specimens of *Sarsia tubulosa* (M. Sars) were collected from the water around Victoria, B. C., Canada and from the dock at the Friday Harbor Laboratories of the University of Washington. They were either maintained in cool, running sea water and exposed to daylight or transferred to a holding tank in a dark room at 8°C. The water in the tank was continuously replenished with fresh, filtered seawater.

For electrical recording, whole or partially dissected animals were pinned down on a silicone elastomer plastic (#184; Sylgard, Dow Corning Corp.) layer in the recording dish. Experiments were done in seawater at 8 to 10°C. Recordings were made from ocelli which had been dark-adapted for at least 15 min. (This time for full dark adaptation was determined experimentally). The extracellular recordings were made with plastic suction electrodes with external tip diameters ranging from 20 to 25 μm and with glass microelectrodes (Ultra Tip, Federick Haer & Co., #30-30-0) with external tips of 5 to 15 μm in diameter. The shaft of the latter was connected to the amplifier input lead by an intermediate piece of polyethylene tube. Both types of suction electrodes were filled with seawater, and both allowed vigorous movements of the preparations without electrical artifacts. The recording electrodes were placed with a micromanipulator in the center of the ocellus (unless otherwise noted) while the indifferent electrode was placed into the bathing medium. By adding isotonic magnesium chloride in suitable portions the amount of muscular activity was reduced.

Electrical responses were fed into a Grass 79C polygraph via capacity-coupled (AC) amplifiers with a half-amplitude fall time constant of 600 msec. The amplified signal was passed through a 5 Hz low pass filter and displayed concurrently on a storage oscilloscope (Tektronix 5111).

For photo-stimulation, light from a tungsten-halogen lamp (150W/120V) was passed through a camera shutter and brought by a single optical fibre to an evenly illuminated spot (approximately 100 μm in diameter), covering the entire retina. The intensity of the light stimulus was controlled by calibrated neutral density filters (Zeiss) placed in the beam beyond the shutter. An optical fibre placed beyond the neutral density filters led to a photocell which monitored the light stimulus. For light measurements a QSL-100 Laboratory Quantum Scalar Irradiance Meter was used. The unattenuated light intensity, measured at the level of the ocellus, was equivalent to 0.7×10^{13} quanta $\cdot$ sec$^{-1} \cdot$ cm^{-2}. Intensities (I) of light stimuli are expressed in log units relative to this value (*e.g.* for a full intensity stimulus, log I = 0.0; for a flash attenuated by a factor of 100, log I = −2.0).

Spectral sensitivities of ocelli were determined by measuring the response amplitude (% of maximum) to 60 msec light stimuli at different wavelengths from 350 to 700 nm with constant intensity. The output of monochromatic light (Balzers color filters) at different wavelengths was measured with a quantum meter, and sensitivity measurements were corrected for the differences in energy with calibrated neutral density filters.

Control responses to a stimulus of a given intensity and wavelength were measured at various times during the experiments to check that sensitivity was not changing with time.

RESULTS

Response pattern to brief and long light stimuli recorded from the center (30 μm in diameter) of the ocellus

The ocellus of *Sarsia tubulosa* responded to a brief light flash (log I = 0.0; 10 to 50 msec in duration) with a single transient positive potential change. This on-response had a latency of 35 to 45 msec and a duration of 200 to 300 msec. The amplitude ranged from 40 to 80 μV (Fig. 2A). The on-response often was followed by a small undershoot of variable amplitude, rarely followed by a slow positive deflection. The undershoot became more pronounced in recordings with closely apposed plastic suction electrodes than with the thinner glass electrodes. It also gradually increased when the electrodes were shifted out of the center of the ocellar cup.

The response pattern to long light stimuli (in the range of 1 sec and longer; log I = 0.0) consisted of an initial transiently positive potential change, identical to the on-response to brief light flashes. It was followed by a second slower biphasic pulse of variable amplitude and with a long recovery phase (Fig. 2B). Whereas the on-response remained stable when light flashes were repeated at intervals of 45 sec or more, the biphasic pulse became smaller due to light adaptation. The off-response was a slow positive deflection approximately 1 sec in duration with a latency of 200 msec. This was often followed by series of small high frequency pulses (with durations ranging from 150 to 300 msec) for up to 60 sec (Fig. 2B).

Glass suction electrodes lightly attached to the center of the ocellar cavity

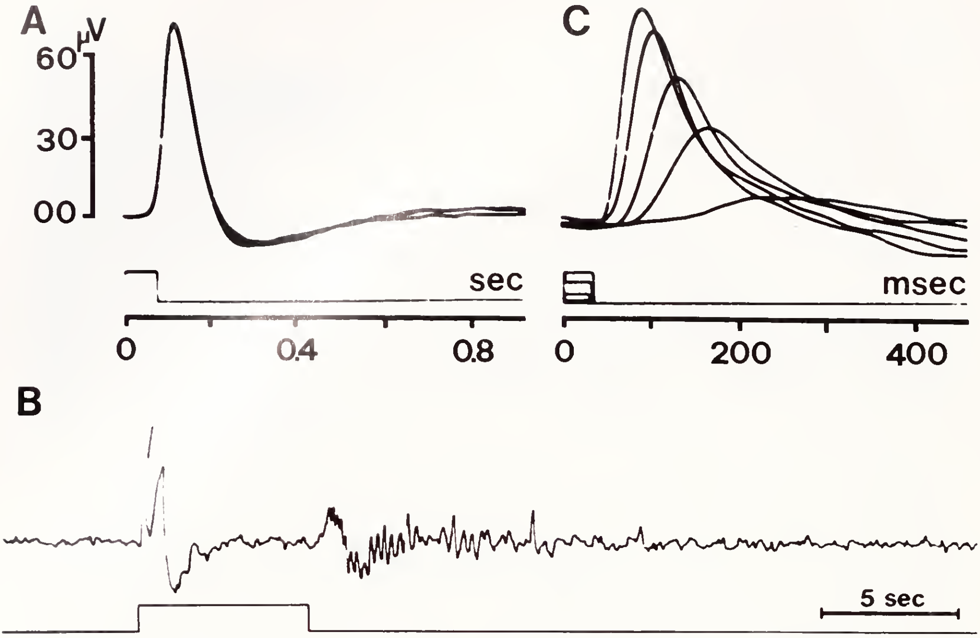

FIGURE 2. Responses to brief and long light stimuli recorded from the center of the ocellus. Lower trace shows signal from photocell monitoring the light stimulus. (A): superimposed oscilloscope traces of on-response to three 60 msec light stimuli (log I = 0.0) at intervals of 30 sec. (B): response pattern to a long light stimulus. The first positive potential change is followed by a second biphasic pulse with a long recovery phase. The off-response is a slow positive deflection followed by a series of high frequency pulses. (C): graded responses of the ocellus to 30 msec light stimuli of increasing intensity. Superimposed oscilloscope traces of responses to five light stimuli. Intensity of successive flashes increases from log I = −3.0 (smallest response); −2.0; −1.5; −0.75 and 0.0 (largest response). Interval between stimuli 60 sec.

recorded the on-response but not the biphasic pulse (Fig. 6B), and the high frequency pulses following the off-response were either very small or missing.

No changes in response pattern to brief and to long light stimuli were recorded from animals kept either in the dark or in the light for at least 12 hours after 90 sec in the dark.

Sensitivity to light and time course of responses

The amplitude of the positive on-response was graded with respect to the intensity of the light. Figure 2C shows superimposed responses to five 30 msec light stimuli of increasing intensity. The relative intensity ranged from log I = −3.0 (smallest response) to log I = 0.0 (largest response). The interval between successive light flashes was 60 sec. The response amplitude reached a maximum at a relative stimulus intensity of about log I = 0.0. Further increases in stimulus intensity usually elicited no further increases in response magnitude. The latency of onset and time to peak of the response decreased with increasing light intensity. The shortest latency recorded for the on-response was 35 msec at log I = 0.0.

Spectral sensitivity

Figure 3 shows the spectral sensitivity curve of the on-response amplitudes expressed as a percentage of the maximum response for ten ocelli against the

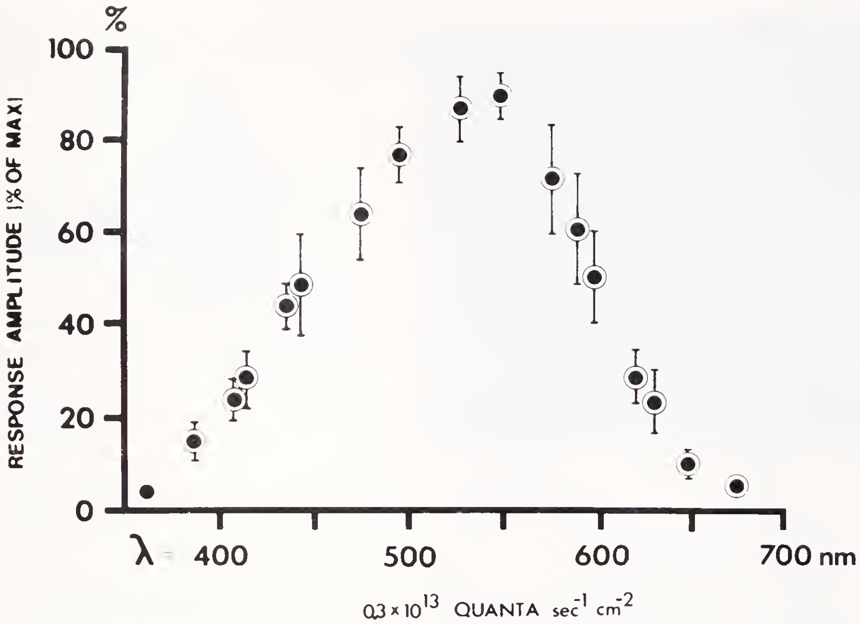

FIGURE 3. Spectral sensitivity of the ocellus of *Sarsia tubulosa*. Mean value (⊙) of response amplitudes expressed as a percentage of the maximum response for ten ocelli against the different wavelengths (nm). Error bars indicate standard deviation.

different wavelengths (nm). Changes of the maximum response amplitudes during each series were less than 5%. The light intensity measured at the level of the ocellus was 0.3×10^{13} quanta·sec^{-1}·cm^{-2}. The spectral sensitivity of the ocelli ranged from λ = 363 nm to 675 nm with its maximum around 540 nm.

Changes in response patterns at different sites of the ocellus

Whereas the above described response patterns to brief and long light stimuli were restricted to the center of the ocellar cavity (Fig. 4A), recordings to brief light stimuli (log I = 0.0) from sites adjacent to the center showed smaller on-response amplitudes. In fact, the amplitude of the positive on-response gradually decreased with increasing distance of the recording electrode from the center. At the periphery of the pigmented cup the on-response could not be recorded. Beyond the edge of the pigmented ring which surrounds the ocellar cavity a negative on-response was recorded (Figs. 4B, C). This negative response increased with distance, reaching a maximum at 60 to 70 μm from the center of the ocellus. A small negative response could be recorded as far as 100 to 120 μm from the center (Fig. 4D).

To analyze whether the two on-responses of opposite polarity were due to the same receptive structure, responses to light stimuli were recorded simultaneously from different sites of the ocellus. Figure 5A shows superimposed oscilloscope traces to three 30 msec light stimuli (log I = 0.0) at intervals of 60 sec. One glass suction electrode was lightly attached to the center of the ocellus while the other was attached laterally to the optic ganglion (approximately 90 μm apart). In all cases, the positive and negative deflections induced by light stimuli were correlated with each other (Fig. 5A). If the electrodes were attached to the optic ganglion at opposite sites (approximately 50 and 70 μm from the center) the latency of onset and time to peak of the negative events was proportional to the distance between

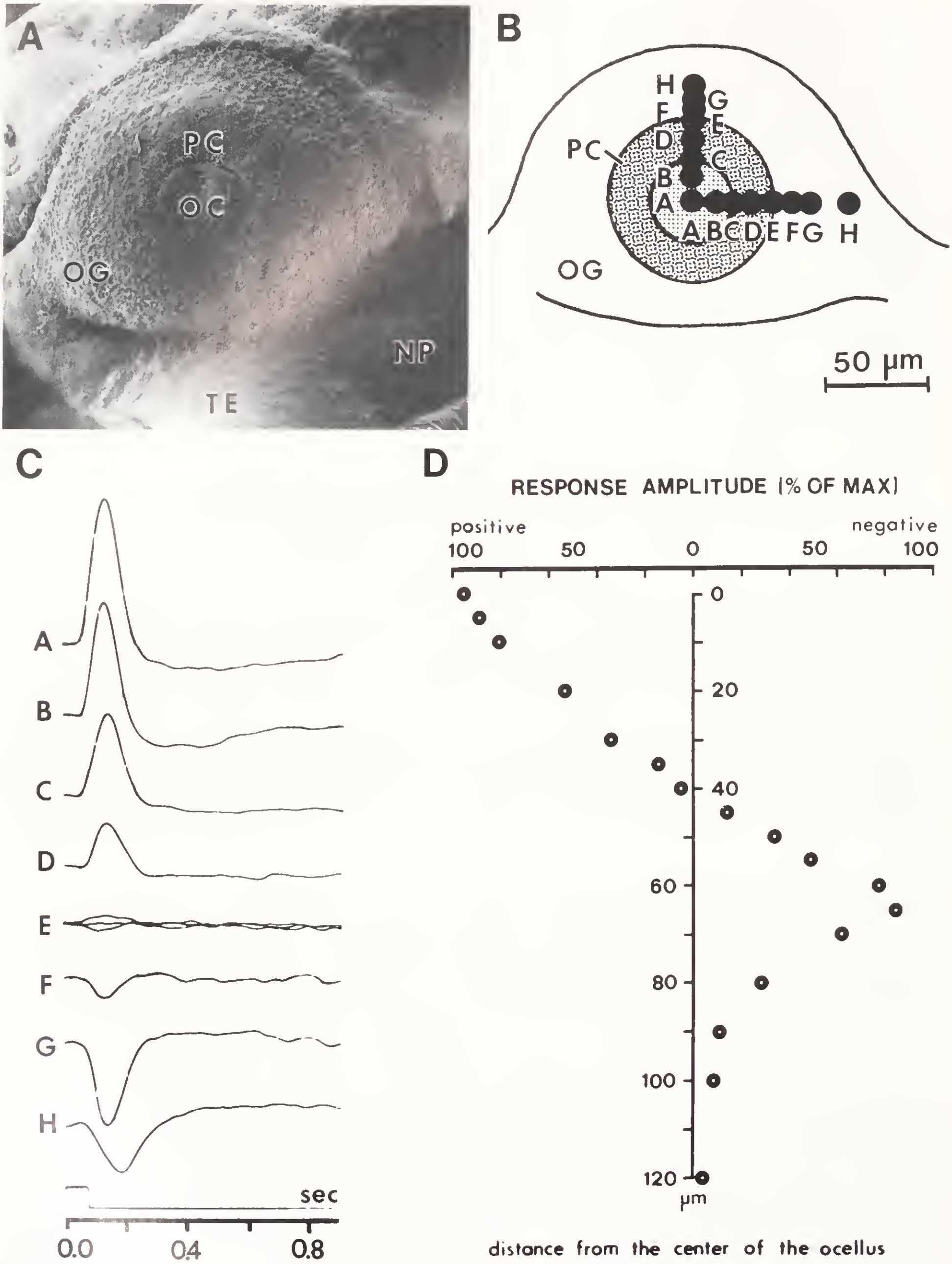

FIGURE 4. Structure of ocellus and summary diagram of changes in responses to brief light stimuli (log I = 0.0) at different sites of the ocellus. (A): scanning electron micrograph of the ocellus of *Sarsia tubulosa.* NP, nematocyst pad; OC, ocellar cavity; OG, optic ganglion; PC, pigmented cup; TE, tentacle. Same magnification as in B. (B): diagram of the ocellus. Each black dot represents the approximate site of a recording electrode. Their distribution pattern is circular. (C): sequence of records, A, B, . . . H, correspond with the sites labelled in B. (D): plot of peak response expressed as a percentage of the maximum response against the horizontal distance from the center of the ocellus. Each point is the average of three to eight measurements.

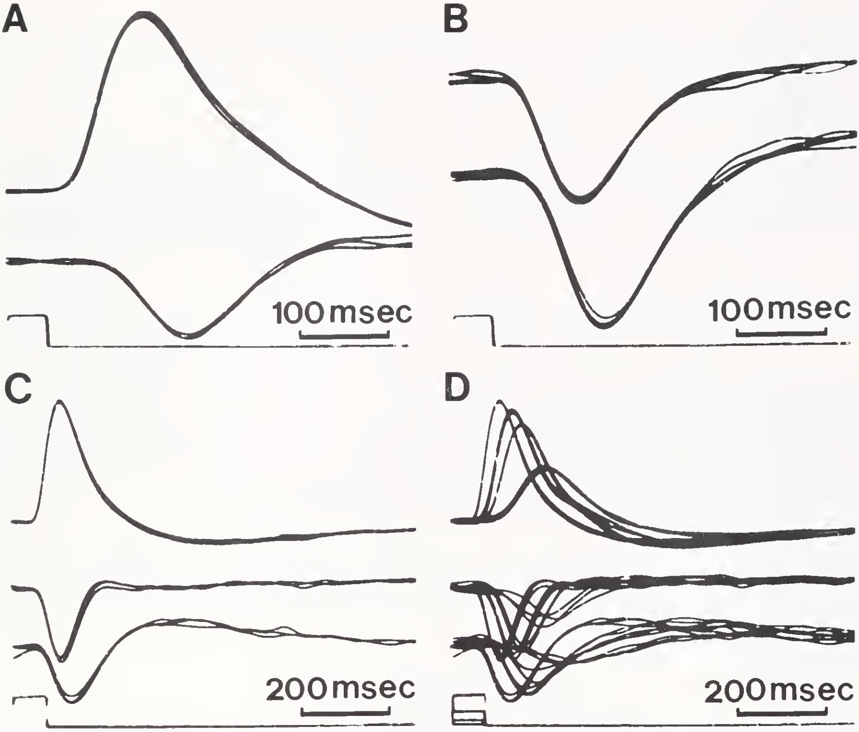

FIGURE 5. (A): superimposed oscilloscope traces of responses to three 30 msec light stimuli at intervals of 60 sec. Records are from the center of the ocellus (positive response) and from the optic ganglion (negative response). (B): superimposed oscilloscope traces of responses to five 30 msec light stimuli. Records of two electrodes attached to the optic ganglion at opposite sites, approximately 50 μm (upper trace) and 70 μm (lower trace) from the center of the ocellus. (C): superimposed traces of responses to three 70 msec light stimuli. One electrode is placed in the center of the ocellus (upper trace) and two electrodes are attached to the optic ganglion, approximately 60 μm (middle trace) and 80 μm (lower trace) from the center. (D): superimposed traces of responses to light stimuli with decreasing intensity. Intensity of successive flashes decreases from log I = 0.0 (largest response); −1.0; −1.5; −3.0 (smallest response). Interval between stimuli 60 sec. Same setting of electrodes as in C. Bottom trace in A, B, C and D shows signal from photocell monitoring the light stimulus. Time measured from beginning of light flash.

the electrodes and the center of the ocellus (Fig. 5B). Recordings with three electrodes, attached to the center and to two different sites of the optic ganglion (approximately 60 and 80 μm from the center; Fig. 5C, D), also demonstrated striking correspondence in changes of response amplitudes to light stimuli of full intensity (Fig. 5C) and to stimuli with decreasing intensity (Fig. 5D).

To compare the response patterns from different sites to brief and long light stimuli, recordings were made simultaneously from the center of the ocellus and from the optic ganglion. Whereas the on-response to brief light stimuli recorded from the ocellus sometimes was followed by a slow positive deflection of variable amplitude, its counterpart recorded from the optic ganglion never included a comparable second event following the slight overshoot of the initial negative potential change (Fig. 6A). The response pattern of the optic ganglion to long light stimuli, however, resembled a reflected image of the response pattern recorded from the center of the ocellar cavity (Fig. 6B), except that the off-response recorded from

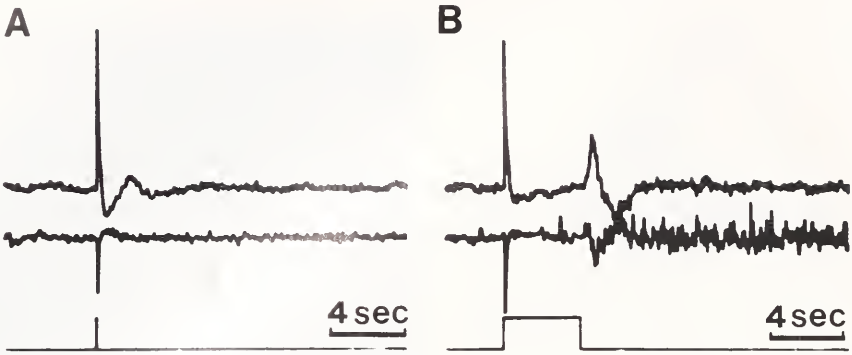

FIGURE 6. Comparison between different response patterns to brief and long light stimuli. (A): recordings from the center of the ocellus (upper trace), and from the optic ganglion (lower trace) to a brief light stimulus (log I = 0.0). (B): same recording sites. Response pattern to a long light stimulus. The high frequency pulses following cessation of illumination recorded from the optic ganglion (lower trace) are conspicuous by their absence in recordings from the center of the ocellus (upper trace). Bottom trace in A and B shows signal from photocell monitoring the light stimulus.

the optic ganglion was succeeded by a series of high frequency pulses which were absent in the center of the ocellus under identical recording conditions (glass suction electrodes).

DISCUSSION

The electroretinogram (ERG) recorded from the center of the ocellus of *Sarsia tubulosa* has a characteristic positive potential change at the onset of illumination, followed by a slower biphasic pulse with a long recovery phase and a positive off-response succeeded by a series of high frequency pulses. This response pattern is strikingly similar to the ERG from the ocellus of the jellyfish *Polyorchis penicillatus* (Weber, unpublished). The most conspicuous features of the on-response are: (1) the relation between its latency and the intensity of the light stimulus, and (2) the graded amplitude with respect to the light intensity and to photic stimulation with different wavelength at constant intensity. The ocellus is most sensitive to blue-green and green light. The spectral sensitivity curve ranges from 363 nm to 675 nm with a maximum around 540 nm.

The use of glass suction microelectrodes with small tip diameters allows splitting of the ERG into component responses and mapping of changes in the ERG at different sites inside and outside the pigmented cup of the ocellus. The results suggest that the positive on-response is mainly due to photo-induced receptor potentials of photoreceptors, and that the presumed receptor potential originates at the center of the ocellar cavity and propagates in all directions. The amplitude of the response decreases with increasing distance from the center. Beyond the edge of the pigmented cup, it increases again but with opposite polarity, then decreases again (Fig. 4). It is still to be determined with correlative work at the intracellular level whether a reverse of the sign of photocurrent along photoreceptors, similar to that found in retinae of vertebrates, cephalopods, and invertebrates (Hagins, *et al.*, 1962; Penn and Hagins, 1969; Shaw, 1972), is responsible for the response patterns of opposite polarity. The physiological observations, however, suggest that *Sarsia*'s photoreceptors hyperpolarize to light as vertebrate receptors do, and that they conduct decrementally.

Histological observations on the ocellus of *Sarsia* show that the cell bodies of photoreceptors lie at various levels behind the ocellar cup, up to 60 μm from the ocellar cavity. The proximal axons of the bipolar receptor cells group together to form a pair of optic nerves (Singla and Weber, in preparation) which encircle the tentacle base and enter the tentacular ganglion (Mackie, 1971). Synapses are common at the optic ganglion between receptor cell bodies, between axons and receptor cell bodies, and among axons (Fig. 1). In this context it is of special interest that in most experiments in which fine glass suction electrodes were used, the series of high frequency pulses following the off-response can only be recorded from the optic ganglion (Fig. 6B). These high frequency pulses probably result from activity in the nervous plexus of the optic nerves following cessation of illumination. It is uncertain, however, whether these post-stimulus fast potentials are identical to preswim pulses recorded from *Sarsia* (Passano, 1976).

The ERG can be recorded out to the edge of the optic ganglion. No responses to light stimuli are recorded from the nematocyst pad and the exumbrella surrounding the optic ganglion. Recordings from the tentacular ganglion, which is situated below the tentacle base, do not show any activity directly correlated with the ERG. It is still to be determined whether "swimming" in *Sarsia* is triggered by the photo-induced responses via direct neuronal pathways (optic nerves) between receptor and effector organ, as appears to be the case in other hydromedusae (Yoshida and Ohtsu, 1973; Anderson and Mackie, 1977).

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DISTRIBUTION OF SUBCELLULAR BIOLUMINESCENT SOURCES IN A DINOFLAGELLATE, *PYROCYSTIS FUSIFORMIS*

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ABSTRACT

Pyrocystis fusiformis exhibits rhythmic changes in the distribution and mechanical excitability of subcellular bioluminescent sources. Bioluminescence in night-phase cells can be stimulated either mechanically or by low pH and originates from microsources found throughout the cytoplasmic layer surrounding the large central vacuole. Microsources are weakly fluorescent and probably correspond to 0.5 μm or smaller cytoplasmic inclusions. With the onset of day phase, bioluminescence becomes mechanically inexcitable but responds to acid stimulation. Microsources disappear from the cell periphery during early day phase and all luminescence originates from the perinuclear region. In late day phase, bioluminescence originates both from the perinuclear region and from microsources in the periphery. However, luminescence remains mechanically inexcitable until the onset of night phase. Cells maintained in darkness exhibit the same rhythmic changes in mechanical excitability and development and disappearance of the perinuclear luminescence, except that microsources do not disappear from the periphery of early day-phase cells without a light induction period. Mechanisms which might underlie the rhythmic changes in bioluminescence distribution and mechanical excitability are proposed.

INTRODUCTION

Identification of particulate light sources (microsources) in bioluminescent cells is needed to understand mechanisms and regulation of light emission. Microsources have been tentatively identified in a number of bioluminescent cells (Sweeney, 1980), but confirmation at the ultrastructural level is lacking except in the firefly (Hanson *et al.*, 1969; Case and Strause, 1978) and the scaleworm (Bassot and Bilbaut, 1977). No dinoflagellate microsource has been positively identified. The phagotrophic dinoflagellate, *Noctiluca miliaris* has figured heavily in such studies because its individual microsource luminescence is readily seen by image intensifier microscopy (Eckert and Reynolds, 1967). However, correlation of *Noctiluca* microsources, as seen by image intensification, with subcellular structures, as identified by other techniques, has not proceeded beyond their correlation with similarly distributed, 1.5 μm or smaller, fluorescent particles in the peripheral cytoplasm (Eckert and Reynolds, 1967).

Unlike *Noctiluca*, which has no daily cycle of luminescence capacity, *Pyrocystis fusiformis* exhibits a circadian oscillation in stimulable bioluminescence (Sweeney, 1981). Night-phase cells luminesce brilliantly upon either mechanical or acid stimulation. Each mechanical stimulus elicits a single flash, the summation of all the

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Abbreviations: CT, circadian time; d, largest cell diameter; DD, 24 h dark cycle; FF, first flash; IIT, image intensifier; l, length; LD, light-dark cycle; PMT, photomultiplier tube; SF, subsequent flash; SRU, stimulus recording unit; TMSL, total mechanically stimulated luminescence.

microsource microflashes. During a first flash (FF) the microsources flash synchronously, while they flash asynchronously during subsequent flashes (SFs) (Widder and Case, 1982). Total light output from acid-stimulated night-phase cells is a prolonged glow representing the summed activity of asynchronously flashing microsources. Day-phase cells are only weakly luminescent upon acid stimulation and are essentially unresponsive to mechanical stimuli (Sweeney, 1981; Widder and Case, 1981). Since our preliminary image intensifier studies revealed that the daily cycle of luminescence capability was associated with the appearance and disappearance, or movement, of microsources, we concluded that a detailed study of this phenomenon might be a step towards identification of microsources. In this paper, the properties of microsources as seen by image intensifier microscopy are described.

MATERIALS AND METHODS

Organisms

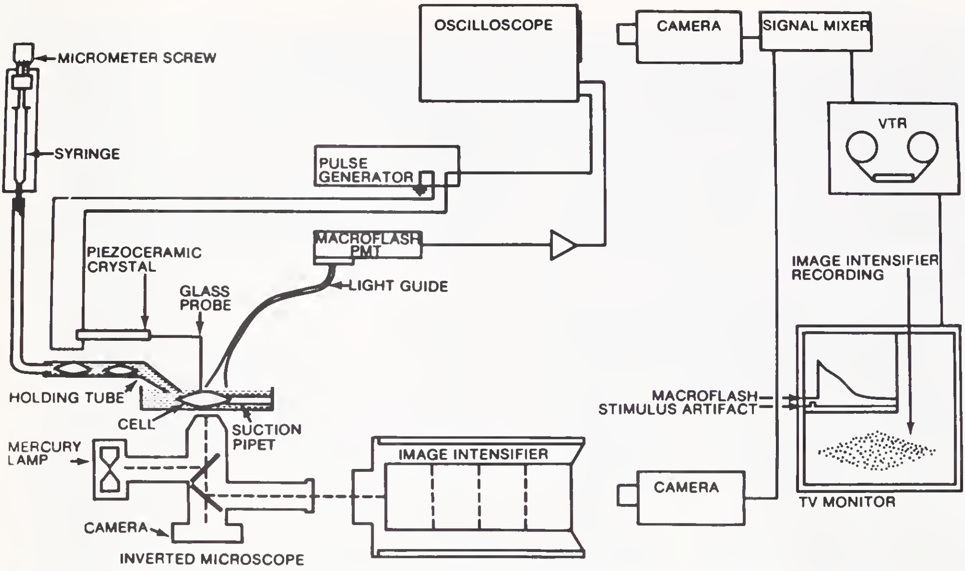
Cultures of *Pyrocystis fusiformis* Murray (No. 5M6, UCSB Algal Culture Collection), and *P. noctiluca* Murray were isolated by Dr. B. M. Sweeney. Unialgal cultures were grown at 20°C under cool white fluorescent lamps at about 500 $\mu W \cdot cm^{-2}$ on a 12:12 LD cycle. The culture medium consisted of sterilized, filtered seawater plus soil extract and "f/2" nutrient (Guillard and Ryther, 1962) minus silicate.

Cells for image intensification microscopy were loaded into glass holding tubes (18 cm long × 2 mm I.D., tapering to 1 mm I.D.) connected by polyethylene tubing to a 1 cc micrometer-controlled syringe (Fig. 1). This system permitted delivery of single cells into the experimental chamber without mechanical stimulation of their bioluminescence. Cells were loaded into the holding tubes during their inexcitable day phase and then returned to the culture chamber and maintained under the standard light and temperature conditions until needed. For cells maintained in darkness (DD), the holding tubes were placed in light-tight containers in the same culture chamber. The effects of light exposure on DD cells were tested by exposing the cells in the holding tubes to the incubator lights (500 $\mu W \cdot cm^{-2}$) for 5, 30 or 60 min beginning at circadian time (CT) 0000 (daybreak) or CT 0200.

Recording methods

Luminescence of a single cell was imaged with an EMI 2001 four stage image intensifier system capable of a radiant power gain of 10^6 (Fig. 1). The cell was held by a glass suction pipet (I.D 80 μm) against the cover slip bottom of a 2 cc Plexiglas chamber filled with filtered seawater and attached to the stage of a Zeiss IM 35 inverted microscope equipped with Plan-Neoflaur 25/0.8 and Planapochromat 100/1.3 oil immersion and 4/0.14 objectives. Images were focused on the image intensifier tube (IIT) photocathode via a Zeiss photochanger (magnification factor 1.6×) and a 6.3× or 16× ocular.

The IM 35 microscope was also equipped for Nomarski interference contrast microscopy with a Planachromat 100/1.25 objective. Nomarski micrographs were taken of a portion of the cell surface (65 μm × 95 μm) using the microscope 35 mm camera for later comparison with bioluminescence and fluorescence recordings made of the same field with the aid of the IIT. Fluorescence excitation was achieved with a 100 W mercury lamp and a Zeiss (48 77 03) filter/reflector combination for reflected violet-selective excitation. This filter set provides narrow band exci-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CELL
SUCTION PIPET
MERCURY LAMP
IMAGE INTENSIFIER
CAMERA
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TV MONITOR
INVERTED MICROSCOPE

FIGURE 1. Experimental apparatus used to localize subcellular bioluminescent and fluorescent sources. A single cell from the holding tube was directed to the suction pipet without stimulating it. Bioluminescence resulting from mechanical stimuli delivered via the stimulator-controlled piezoceramic crystal or by the addition of acid to the seawater bath was recorded by a side window photomultiplier tube (PMT) via a light guide situated in the bath. PMT output was displayed on a storage oscilloscope and the display was recorded on video tape. The image of the cell was focused on the photocathode of the image intensifier tube (IIT) via the inverted microscope. Video recordings of the IIT output phosphor provided spatial and temporal records of subcellular bioluminescent activity which could be simultaneously displayed with the oscilloscope recordings of total light output. Photomicrographs of whole cells (as for Fig. 2) and Nomarski micrographs of cytoplasmic features were made with the internal camera before and/or after IIT recordings of bioluminescence and fluorescence.

tation at the 404.7 nm mercury line and also includes an FT 420 chromatic beam splitter with the associated barrier filter replaced by an interference filter (BP 520–560) to eliminate the intense red chlorophyll fluorescence.

Mechanical excitation was provided by a manipulator-held glass probe (tip diameter 50 μm) driven by a piezoceramic bender (Gulton Industries) controlled by a Grass S44 pulse generator. Acid stimulation of bioluminescence was achieved either by local micropipet application of 0.1 *M* acetic acid to the cell surface, or by addition of 0.05 cc of 4 *M* acetic acid or 23 *M* or 6 *M* formic acid to the 1.5 cc seawater bath.

A fiber-optic light guide built into the chamber wall led to an EMI 9781A photomultiplier tube (PMT) to register total light output on a storage oscilloscope. The image intensifier output was recorded with a video camera equipped with a Newvicon® tube and an f 0.95 lens. A second video camera recorded a video frame counter and the oscillographic display of the PMT recording of luminescent activity. A composite image of the two video recordings was formed by a Shintron Model 366 Special Effects Generator and recorded on a Sony VO-2610 videocassette recorder. A detailed description of the apparatus is presented elsewhere (Widder and Case, 1982). The time resolution of the video records was 16.7 ms/field.

To compare light micrographs with the video recordings of the same field, Polaroid micrographs were made from a video screen during real time playback.

For acid stimulation sequences, in which the microsources flash asynchronously, the exposures were as long as the stimulus sequence in order to record flashes from as many of the microsources as possible. The Polaroid photographs of the bioluminescence and fluorescence were then enlarged to 8 × 10 inch prints for comparison with the Nomarski photographs of the same field. A hemocytometer grid was photographed by both methods to correct for distortion introduced by the image intensifier and to adjust the enlargements to the same magnification.

Microsource counts were made from the video recordings using a video screen with a magnification of 9000 at the highest magnification used (100× objective and 6.3× ocular). Spots identified as microsources were traced on a transparent overlay as the video record was advanced one frame at a time. Night-phase cells were stimulated either mechanically or with acid and the sequence was studied until the luminescence was exhausted or the cell had moved. Study of prolonged luminescent activity made it possible to distinguish single from clumped microsources as the microsources flashed asynchronously. The number of microsources seen within an area (2500 μm^2 or 1849 μm^2) at the cell surface was related to the total cell surface area calculated as $0.5 \cdot \pi \cdot d \cdot [0.596 \cdot l + (d^2 + 0.493 \cdot l^2)^{0.5}]$ (Widder & Case, 1981). This formula fits the shape of stage I and II cells. In stage III cells the plasma membrane pulls away from the cell wall to form a constriction around the center (nuclear) region of the cell which complicates the surface area calculations. Therefore we only used stage III cells in which the central constriction was minimal.

Mechanical excitability of LD and DD cells was tested with the stimulus recording unit (SRU) described in Widder and Case (1981). Briefly, single stage II cells were loaded into fluoroplastic tubes during their mechanically inexcitable day phase. The cell-containing tube was placed in fixed relation to a photomultiplier tube and suprathreshold mechanical stimuli of fixed strength were applied directly to the tube at a frequency of 0.33 pps with a stimulator-controlled solenoid. The cell was stimulated to exhaustion and the integrated light output of the first flash (FF), the total mechanically stimulated luminescence (TMSL), and the total number of flashes produced were recorded.

The cell cycle of *P. fusiformis*, originally described by Swift and Durbin (1971), takes about 5 days in our cultures. Stages in the life cycle, identified according to the morphological criteria of Widder and Case (1981), are illustrated in Figure 2. Note that "stage" refers to cell morphology and "phase" refers to time of day.

RESULTS

Distribution of bioluminescence during the daily cycle

To determine the intracellular distribution of microsources in a normal light-dark environment, 192 cells from different stages in the life cycle were examined with the IIT through the 24 h cycle. The five morphological stages of the life cycle in night phase and early day phase are shown in Figure 2, along with the luminescence patterns in response to acid stimulation. Changes in the pattern of luminescent activity over the 24 h cycle were considered in 5 phases: 1) night 2) early day 3) late day 4) day to night transition ("dusk") 5) night to day transition ("dawn"). Results are summarized in Table I and are briefly discussed below.

Night-phase cells (CT 1400–2200)

Both mechanical and acid stimulation revealed generally uniform distribution of microsources in the peripheral cytoplasm (Fig. 2). The cytoplasm forms a thin

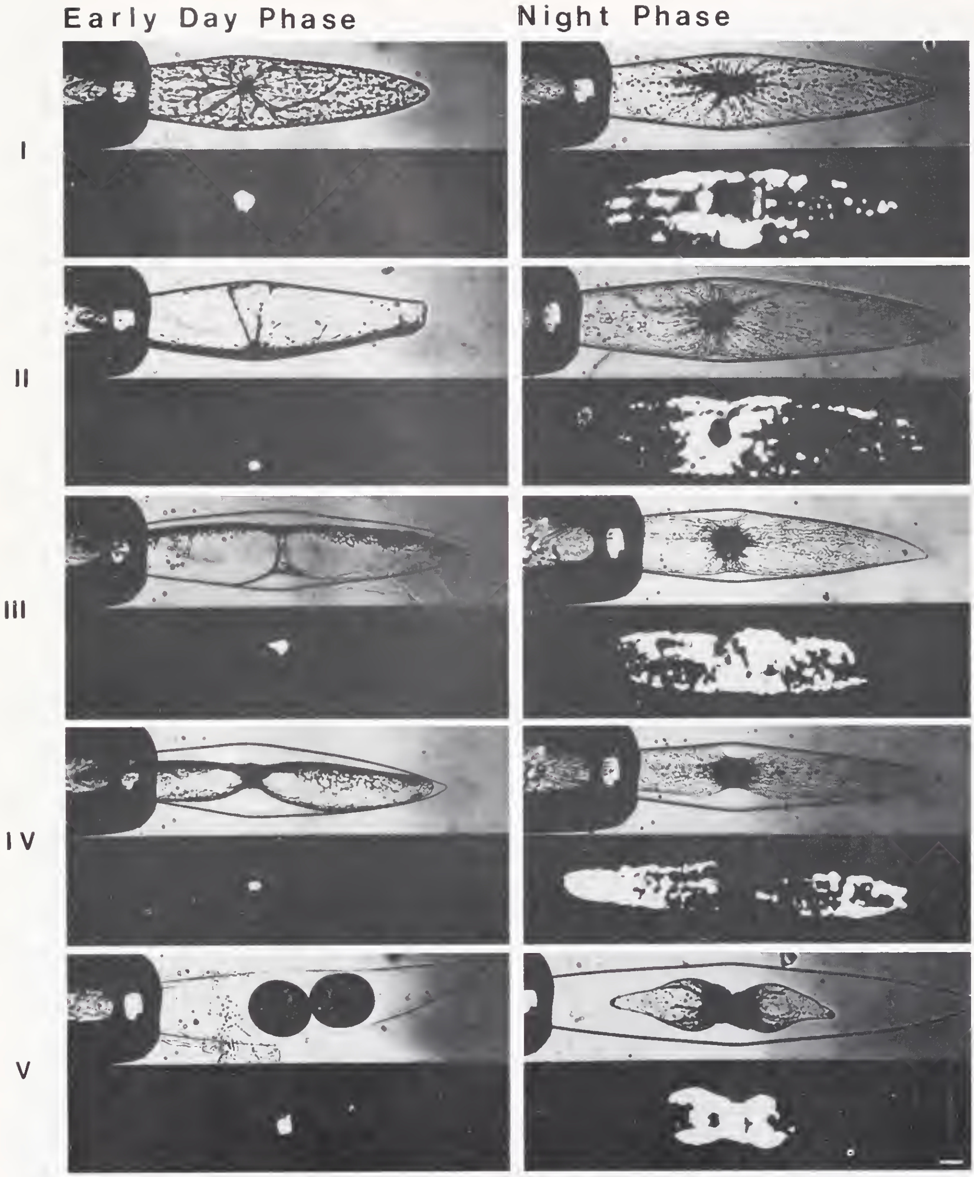

FIGURE 2. Distribution of subcellular luminescence in the five morphological stages (I–V) of cells in early day phase and night phase. Each of the 10 cells shown was positioned on the suction pipet, seen on the left, and photographed. The cell was then stimulated with acid and a video-IIT record of the resulting bioluminescence was made. Polaroid photographs of the video screen made during real-time play back are shown below each of the photomicrographs. In the night-phase stage I and II cells shown here, the nucleus was on the side of the cell closest to the objective and corresponds to the dark region seen in the IIT micrographs of bioluminescence. In the night-phase stage III cell, the nucleus was on the far side of the cell and no dark region was apparent. The night-phase stage V cell exhibits the morphology seen before aplanospore formation. For the two early day-phase aplanospores shown, the one on the left had its nucleus closest to the objective, while the nucleus of the right-hand aplanospore was on the far side of the cell, which accounts for its apparently dim perinuclear glow. Note that the chloroplasts which cover the surface of day phase cells migrate into a tight clump in the perinuclear region during night phase (Swift & Durbin, 1971; Sweeney, 1981). Scale, 50 μm.

TABLE I

Distribution of bioluminescence in cells maintained in normal LD cycle, stimulated by 4 M *acetic acid.*

Circadian time	N	Perinuclear luminescence		w/ss*	Cytoplasmic microsource luminescence	
0000–0100 (Dawn)	12	12/12	Stage I & V—diffuse Stages II–IV—well defined	1/12	12/12	Stages I & V—evenly dispersed Stages II–IV—in bands and bridges
0100–0200	17	17/17	Stages I & V—diffuse Stages II–IV—well defined	3/17	10/17	Became rare, disappearing near end of hour Stages I & V—evenly dispersed Stages II–IV—in bands and bridges
0200–0600	40	40/40	Stages I & V—diffuse Stages II–IV—well defined	17/40	0/40	
0600–0900	26	26/26	Well defined	8/26	21/26	Rare microsources around perinuclear zone; generally in cells w/o ss
0900–1000	9	9/9	Well defined but large	0/9	9/9	More, mostly around nuclear zone
1000–1100	11	11/11	Glow extended into bridges in 3 cells	0/11	11/11	More
1100–1200 (Dusk)	16	16/16	Glow spread into bridges and bands	0/16	11/16	Stage I—higher concentration around nucleus Stages II–IV—in bands and bridges
1200–1300	16	16/16	Diffuse	0/16	16/16	Higher concentration around nucleus. Otherwise evenly dispersed over cell surface
1300–1400	3	3/3	Very diffuse	0/3	3/16	Evenly dispersed
1400–2200	28	0/28			28/28	Evenly dispersed
2200–2300	7	0/7			7/7	Evenly dispersed w/mech stim w/acid more concentrated around center
2300–2400	7	6/7	Diffuse	0/7	7/7	Evenly dispersed except for one cell at end of hour with microsources concentrated in bands

* With microsource-like substructure.

Ratios in Tables I–III indicate what proportion of the cells tested (N) in a given time period responded with the type of luminescence designated by the column heading.

layer (less than 5 μm thick) over the surface of the vacuole with a thickened area around the nucleus (Sweeney, 1980; Sweeney, 1981). In stages II and III, two prominent cytoplasmic bridges extend from the perinuclear zone across the vacuole to the cytoplasm on the opposite side of the cell. Focusing through an acid-stimulated stage II cell (Fig. 3) showed microsources only within the thin layer of cytoplasm around the vacuole and in the cytoplasmic bridges. The cytoplasm between the nucleus and the cell wall was not luminescent (Figs. 2 and 3), possibly because the chloroplasts migrate into this area at night (Sweeney, 1981). Luminescence in night-phase cells appeared to originate only from the microsources, since no diffuse luminescence was seen at $10^6\times$ image intensifier gain.

Early day-phase cells (CT 0200–0600)

Microsources were not visible in the peripheral cytoplasm and all luminescence was confined to the perinuclear zone (Fig. 2). Cells were mechanically inexcitable throughout the day phase except rarely when one or two flashes occurred in the perinuclear zone (Fig. 4c). These were extremely dim (about 20,000 times less than the TMSL from a night-phase cell), brief (about 60 ms with rise time of about 10 ms, as compared with an FF which has a similar rise time but lasts more than 200 ms), and appeared as a diffuse emission around the nuclear region, lacking a clearly defined boundary. Unlike mechanical stimulation, acid stimulation consistently in-

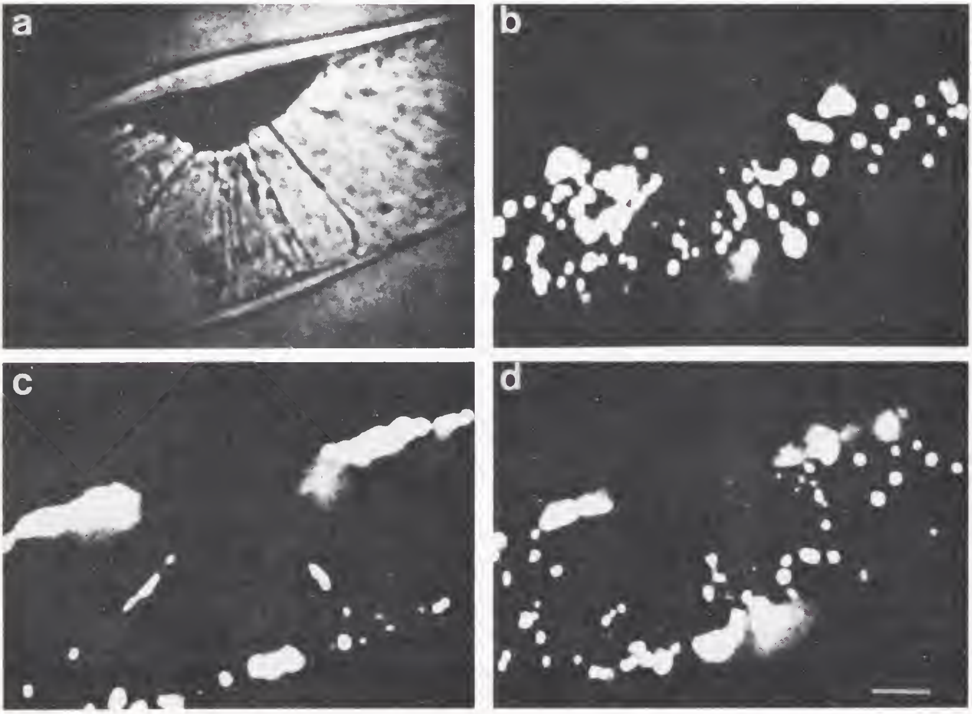

FIGURE 3. Three levels of focus in a stage II night-phase cell during acid stimulation. The cell was backlit (a) and photographed through the IIT prior to stimulation with acid. In (b) the focus is on the near side of the cell. Focusing to midpoint of the cell demonstrates the absence of microsources in the central vacuole except for those seen in the two cytoplasmic bridges which cross the vacuole (c). In (d) the focus is on the far side of the cell. Scale, 50 μm.

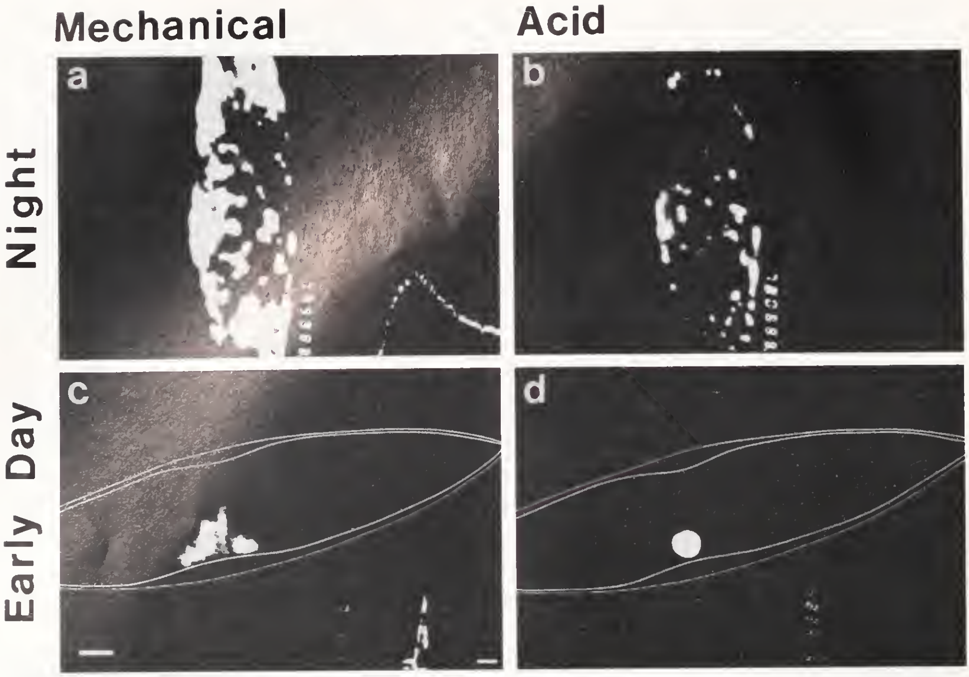

FIGURE 4. Mechanical and acid stimulation of a stage II night-phase (CT 1415) cell (a and b) and a stage III early day-phase (CT 0530) cell (c and d). The outline of the day-phase cell was traced in white from a photograph of the same backlit cell. The night-phase cell received 12 mechanical stimuli and responded with one FF and 11 SFs. The response shown (a) was the eleventh SF. It was immediately followed by acid stimulation (b), which appears dimmer because the luminescence was partially fatigued. The day-phase cell received 5 mechanical stimuli, but apparently responded only to the first stimulus (c). Acid stimulation (d) followed mechanical stimulation as in the night-phase cell. The PMT recordings of the mechanically stimulated flashes are shown at the bottom right of (a) and (c). The time bar in (c), 50 ms, applies to both oscillographic records. Scale, at lower left, 50 μm.

duced luminescence from day-phase cells. This was about 10 times dimmer than from night-phase cells (Sweeney, 1981), and appeared as a bright, clearly defined glowing spot near the nuclear zone. The perinuclear glow appeared either round, diameter about 30 μm (Figs. 5c and f), elliptical or saddle-shaped, about 60–90 × 40–70 μm (Figs. 5a and d). Depending on the orientation of the cell, the ellipse surrounded one or two dark areas probably representing the horseshoe-shaped, non-luminous nucleus. In the round form, the glow frequently exhibited an active, microsource-like substructure, appearing to "boil" (Figs. 5g–i). The round glow with substructure or the elliptical perinuclear glow appeared in all stages and frequently occurred sequentially in the same cell, with the elliptical glow preceding the appearance of substructure as in Figures 5a and c, or *vice versa.* At the onset of acid stimulation, individual sources could be seen that were easily distinguished from background glow (Fig. 5g) and their size and flash duration appeared equivalent to microsources seen in night-phase cells.

Late day-phase cells (CT 0600–1000)

Microsources appeared in the periphery with the greatest density around the perinuclear zone. As microsources appeared, fewer cells exhibited substructure in

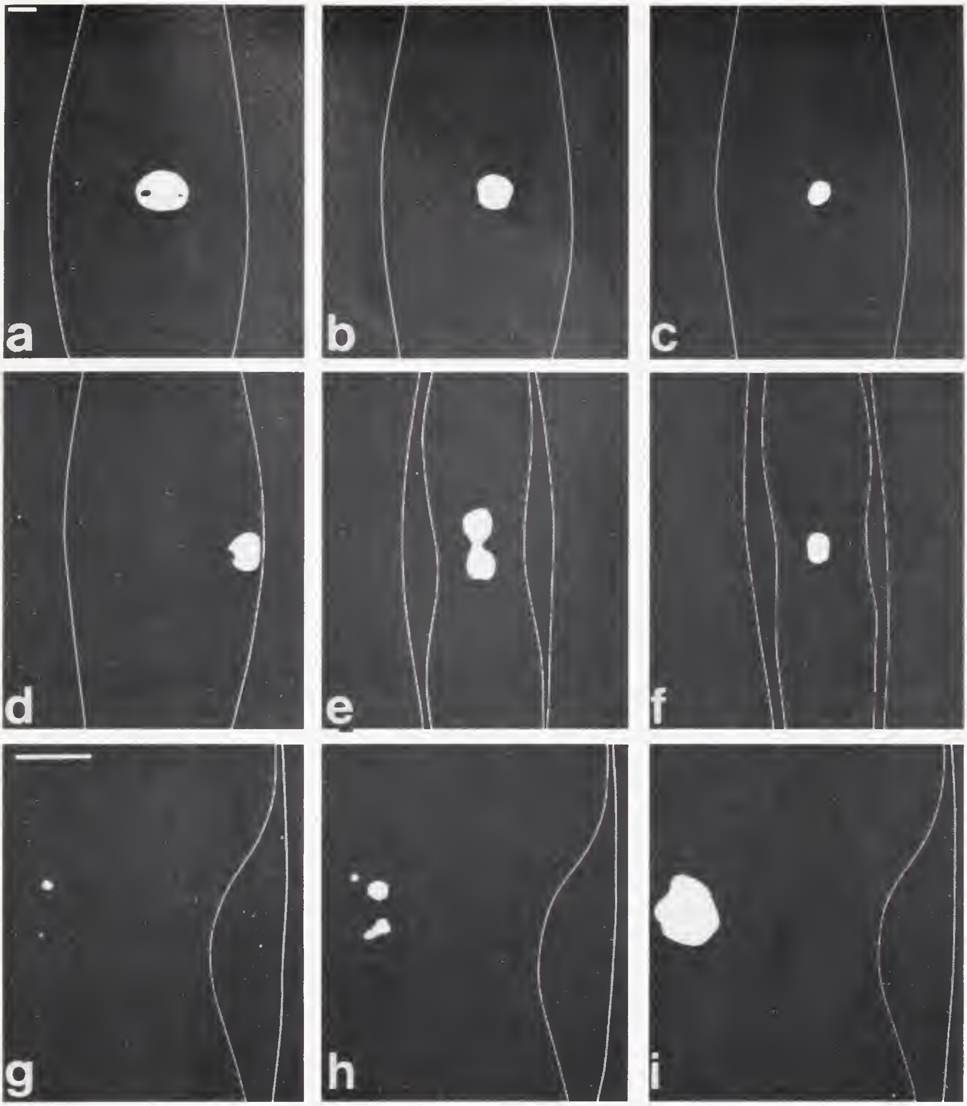

FIGURE 5. Forms of perinuclear glow observed in day-phase cells. White outlines are traced from backlit imaging of each cell prior to acid stimulation. (a–c) Transition from the elliptical perinuclear glow to the round perinuclear glow with substructure in an acid-stimulated stage II cell. Time between (a) and (b), about 45 sec; between (b) and (c), about 25 sec. When a cell was positioned with the nucleus to one side, only one dark area was seen in the elliptical perinuclear glow (d) compared to the 2 dark areas seen when the cell was positioned so the nucleus was in the center (a). The perinuclear glow in stage IV cells appeared as either a double (e) or single spot (f). (g–i) Development of the perinuclear glow in a stage III cell. Initially luminescence appeared as individual microflashes (g) and within 15 sec developed into round glow with substructure (i), which, in this case, persisted for more than 10 minutes. Scale, 30 μm. Upper scale bar applies to (a–f), lower to (g–i).

the perinuclear glow. The acid-stimulated microflashes had the same appearance as those from night-phase cells except that they occurred only briefly, while the nonstructured perinuclear glow persisted for 10 min or more.

Day-to-night transition phases (CT 1000–1400)

During the two hours (CT 1000–1200) before night phase the nonstructured central glow spread into the cytoplasmic bands and bridges (Fig. 6) and numerous microsources were visible. In stages II, III, and IV they were not evenly distributed over the cell surface as in night-phase cells, but were confined to the cytoplasmic bands. Microsources were also numerous in the cytoplasmic bridges crossing the large central vacuole from the perinuclear cytoplasm to the cytoplasmic band, running along the opposite side of the cell. Microsources of stage I cells covered the whole cell surface with the highest concentration around the nucleus. The microsources became mechanically excitable with the onset of night phase, and the perinuclear glow also appeared to be mechanically excitable. Occasionally, the glow appeared during an FF, an effect perhaps produced by the extremely bright emission of densely packed microsources. The glow was generally not seen in the SFs immediately following the FF. With repeated stimulation, however, a glow persisted around the perinuclear region for several seconds after stimulation ceased. This luminescence arose from densely packed microsources, as in the perinuclear glow with substructure, seen in early day-phase cells. The perinuclear glow was much more apparent with acid than with mechanical stimulation.

Night-to-day transition phases (CT 2300–0200)

From CT 1400 to CT 2300, luminescence from either mechanical or acid stimulation originated only from the evenly distributed microsources. Perinuclear glow became visible 1 h before sunrise (CT 2300) and was also occasionally seen with mechanical stimulation as in day-to-night transition-phase cells. As the cells became mechanically inexcitable during the first hour of light, acid stimulation revealed the microsources to be concentrated in the cytoplasmic bands and bridges of stages II, III, and IV and evenly distributed over the cell surface in stage I cells (Fig. 7). During the second hour of daylight (CT 0100–0200), the microsources became rare and dim and the perinuclear glow became brighter and well defined, except in stage I cells. In these, the microsources remained visible longer and the perinuclear glow assumed an astral appearance without well-defined boundaries. Since stage V cells (reproductive bodies) expand to form the stage I cells (Swift & Durbin, 1971), they might be expected to share many characteristics. Specifically, the perinuclear glow was diffuse, and the microsources remained visible over a longer period.

Effects of DD on microsource distribution

The rhythm of chloroplast migration and mechanically excitable bioluminescence in *Pyrocystis fusiformis* persists for more than 96 hours in darkness (Sweeney, 1981). In contrast, our image intensification analysis revealed that microsources did not disappear from the peripheral cytoplasm if the dark period continued through the ensuing subjective day. This was only apparent with acid stimulation, since the microsources were mechanically inexcitable. Although the microsources did not disappear from the periphery as in normal day-phase cells, the perinuclear glow still developed normally (Table II). Microsource distribution was not as in normal night-phase cells, but rather was identical to that assumed by normal LD cells during the first hour of daylight (CT 0000–0100) (Fig. 8). Acid stimulation revealed microsources in the cytoplasmic bridges and bands of stages II, III, and IV along with a nonstructured perinuclear glow. In stages I and V, the microsources

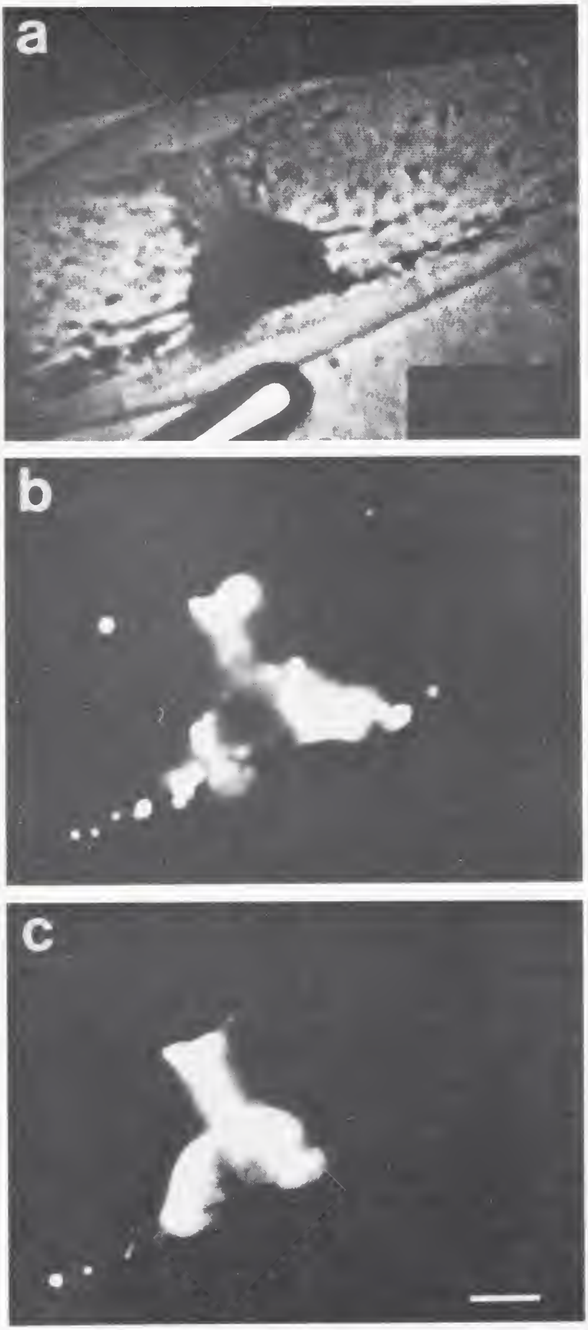

FIGURE 6. Light-to-dark transition phase, stage III cell at CT 1255. (a) Backlit, showing mechanical stimulator probe. (b) Fortieth SF. (c) Response to acid stimulation following 41 mechanical stimuli. Scale, 50 μm.

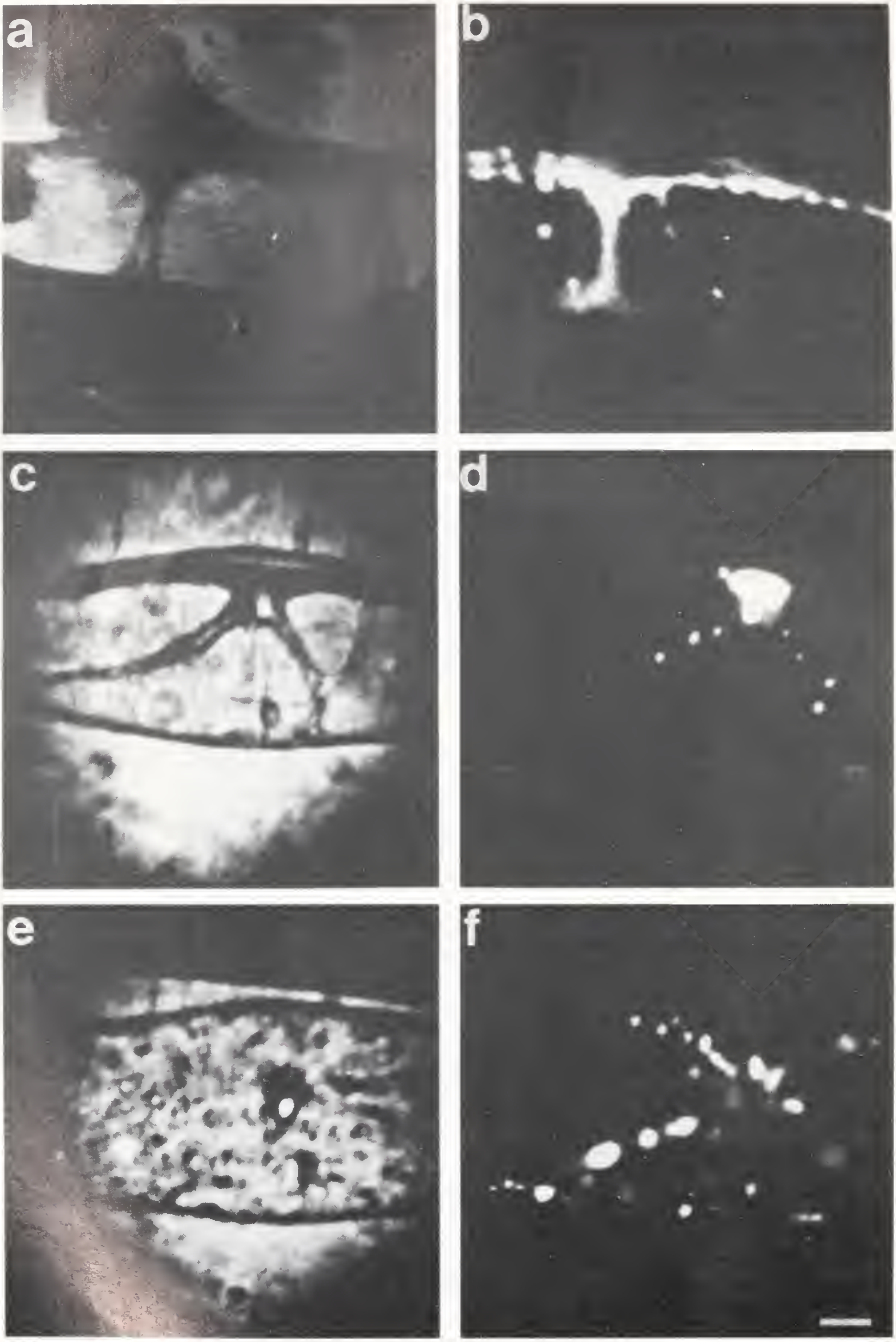

FIGURE 7. Three dark-to-light transition phase cells. Acid stimulated (a) and backlit view (b) of stage II cell at CT 2355. Acid stimulated (c) and backlit view (d) of another stage II cell at CT 0040. Acid stimulated (e) and backlit view (f) of a stage I cell at CT 0110. Microsources in the stage II cells were confined to the cytoplasmic bands and bridges, but were more generally distributed in the stage I cell. Scale, 50 μm.

TABLE II

Distribution of bioluminescence in cells maintained in DD, stimulated by 4 M acetic acid.

Circadian time	N	Perinuclear luminescence	w/ss*	Cytoplasmic microsource luminescence
0000–0100	15	13/15 Spread into bands and bridges in 5 cells	4/15	15/15 Stage I—evenly dispersed Stages II–IV—in bands and bridges
0100–0700	46	36/46 Stages I & V—diffuse Stages II–IV—well defined	0/46	46/46 Stages I & V—evenly dispersed Stages II–IV—in bands and bridges
1100–1300	2	2/2 Stage I—very diffuse Stage IV—spread into bands and bridges	0/2	2/2 Stage I—evenly dispersed Stage IV—in bands
1500–1800	21	0/21		21/21 Evenly dispersed

* With microsource-like substructure.

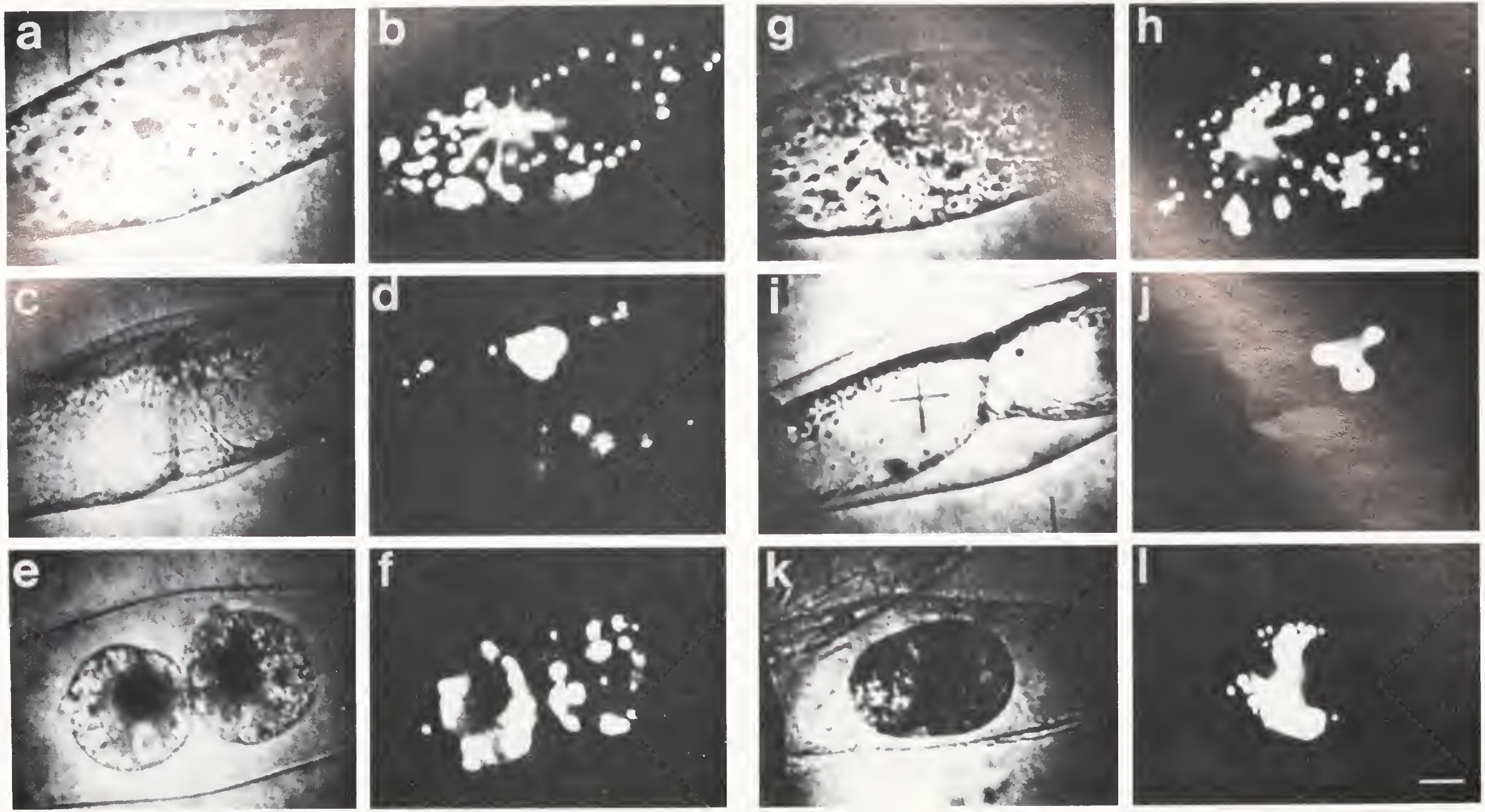

FIGURE 8. Distribution of subcellular luminescence in DD cells (a–f) and cells that received 1 h of light exposure from CT 0000 to CT 0100 (g–l). In each group the backlit cell is shown on the left and its response to acid stimulation is shown on the right. (b) and (h) are typical of the distribution seen in stage I cells under these light regimens; (d) and (j) are typical of the distribution seen in stages II, III, and IV. (e) and (f), a double aplanospore (stage V) and (k) and (l) a single aplanospore. Cells were tested between CT 0300 and CT 0500. Scale, 50 μm.

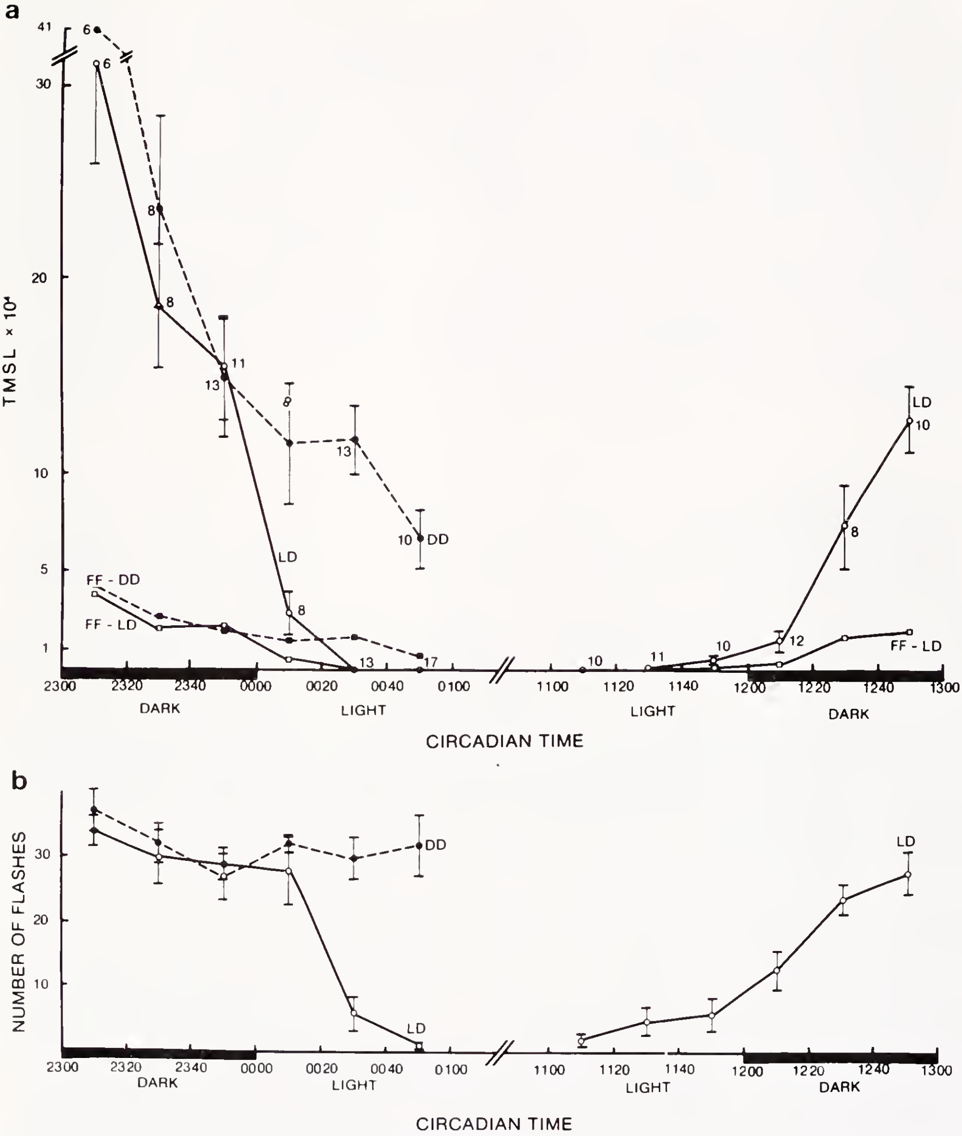

FIGURE 9. TMSL (a) (in arbitrary units) and total number of flashes (b) from transition-phase cells that were mechanically stimulated in the SRU. Dotted lines connect means of DD cells (closed circles) and solid lines connect means of LD cells (open circles). Cells were tested during 6 different dark-to-light and light-to-dark transitions and then means were taken of all cells tested within each 20 min interval. Bars represent standard error. Note that no correction was made for variations in cell size. N values are shown with each mean in (a) and also apply to (b).

remained evenly distributed in the peripheral cytoplasm and the perinuclear glow was diffuse and not located within a well-defined boundary, as in the other stages. During the following night phase of cells left in darkness, the cells became mechanically excitable again, the microsources were again evenly dispersed in the peripheral cytoplasm, and the perinuclear glow was no longer visible.

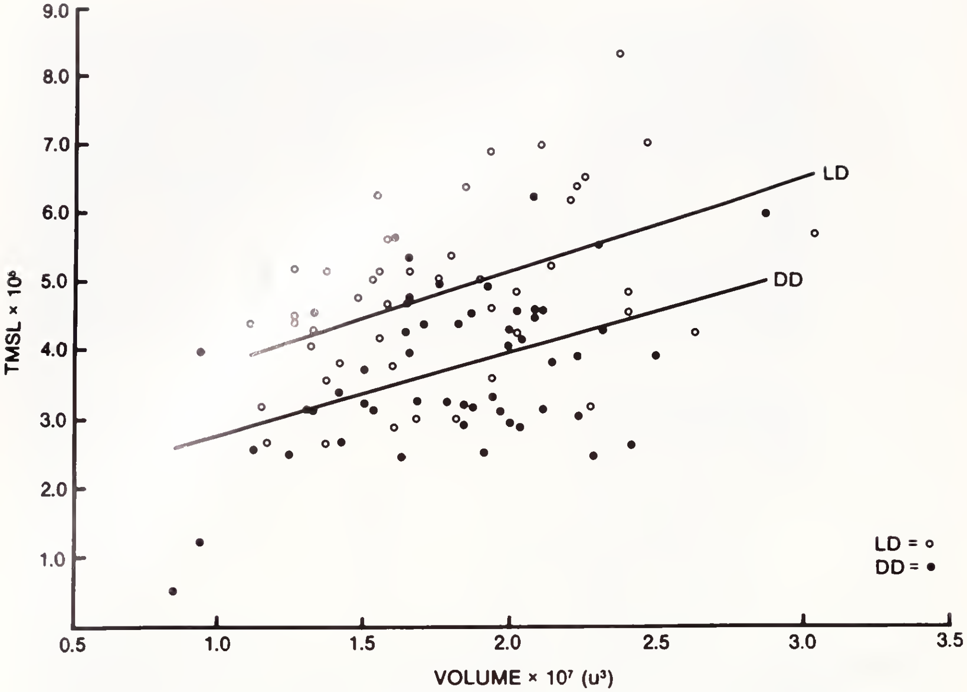

FIGURE 10. Total mechanically stimulated luminescence (in arbitrary units) at CT 1400–1900 plotted as a function of cell volume for 55 night-phase cells maintained under a normal LD cycle (open circles) and 63 night-phase cells maintained in DD (closed circles) for 28 to 33 h prior to testing. The regression of LD cells against cell volume produced the line $y = 0.013x + 245{,}000$ (correlation coefficient $= 0.470$). The regression of DD cells against cell volume produced the line $y = 0.012x + 159{,}000$ (correlation coefficient $= 0.427$).

Effects of DD on mechanically stimulated luminescence

The mechanical inexcitability of persistent microsources in "day"-phase cells left in DD was confirmed in experiments using another method of mechanical excitation. Single cells maintained under LD or DD were mechanically stimulated in the stimulus recording unit (SRU) according to the method of Widder and Case (1981). The total mechanically stimulated luminescence (TMSL) anticipated lights on and lights off (Fig. 9a), as might be expected of a circadian-controlled process. TMSL dropped dramatically in the hour preceding CT 0000 (lights on) and light output ceased less than one hour later. TMSL dropped less dramatically after CT 0000 for cells in DD, but no luminescence was recorded from mechanically stimulated LD or DD cells that were tested between CT 0500 and 0600. Interestingly, the number of flashes a cell could produce did not decrease in anticipation of dawn as did the TMSL. Also, while the TMSL of DD cells decreased in the hour following CT 0000, the number of flashes remained constant (Fig. 9b).

Mechanical excitability of day-to-night transition phase cells was only tested in LD cells which were loaded less than 3 h before testing. DD cells would have had to be maintained in the tubes more than 24 h which greatly increased the variability of the response. However, we did measure TMSL between CT 1400 and CT 1900 for 55 LD cells and 63 DD cells that were maintained in the fluoroplastic tubes between 28 to 33 hours prior to testing (Fig. 10). The regressions of TMSL on cell volume (calculated as $0.133 \cdot \pi \cdot l \cdot d^2$ [Swift and Meunier, 1976]) for each group of cells produced the lines shown in Figure 10 with correlation coefficients of 0.470 and 0.427 for the LD and DD cells, respectively. Although these correlation coefficients were considerably lower than those we have observed in previous experiments (Widder and Case, 1981), they are still significant at the $P = 0.001$ level. The regressions were compared according to the method of Zar (1974, p. 228). No significant difference was found in the variances ($F = 1.216$, df = 45, 49, $P > 0.50$) or the slopes ($t = 0.297$, df = 92, $P > 0.50$), although comparison of elevations did show a significant difference ($t = 4.236$, df = 93, $P < 0.001$), demonstrating that the DD cells that did not receive a normal day phase produced significantly less luminescence than LD cells. The low correlation coefficients probably resulted from the fact that the cells had to be maintained in the fluoroplastic tubes for more than 24 hours prior to testing compared with 4 to 9 hours in our original experiments.

Effects of light exposures on cells maintained in DD

Since the microsources did not disappear from the peripheral cytoplasm of cells maintained in DD, their distribution was evidently directly coupled with the light regimen. This was explored by determining the light duration required to induce their disappearance. Three durations were tested and the results are summarized in Table III. Cells exposed to 5 min of light (500 $\mu W/cm^2$) at the start of the light cycle (CT 0000–0005) were identical to cells maintained in DD except that the microsources appeared more concentrated around the nucleus in stage I cells. Thirty minutes of light noticeably reduced the number of microsources visible with acid stimulation in stages II, III, and IV, and in all stages the microsources were most concentrated around the nucleus. One hour of light exposure at the start of the light cycle (CT 0000–0100) further reduced the number of microsources to a few in the cytoplasm, mostly near the perinuclear zone. However, stage I cells showed little or no obvious reduction in the number of microsources. One hour of light exposure 2 h into the nominal day phase (CT 0200–0300) seemed to be more effective since no microsources were seen in stages II–IV and very few remained in stages I and V.

Numbers of microsources

Estimates of microsource numbers were made by counting the microsources seen to flash within a known fraction of the cell surface area of a night-phase cell. The distribution of the microsources over the entire surface of cells viewed at low magnification showed no obvious regions of high or low concentration except for the small dark area near the perinuclear zone. At the high magnification used to count microsources, however, the microsources appeared in strings and clumps (Fig. 11b). During a first flash (FF) when the microsources flash synchronously (Widder and Case, 1982), it is difficult to distinguish the number of microsources in a clump. During subsequent flashes (SFs) in response to continued mechanical stimulation or during acid stimulation, the microsources flash asynchronously. By observing a sequence of asynchronous flashes, it was possible to distinguish the number of active microsources within a clump. However, the microsources may move along the cytoplasmic strands so that the same microsource may be counted more than once. Counts were made of both mechanically stimulated and acid-stimulated cells. As indicated in Tables IV and V, counts were quite variable and ranged between 1,000 and 13,000 microsources per cell with an overall average of 4,500 (SD

TABLE III

Light effects on bioluminescence distribution in cells maintained in DD, stimulated by 4 M acetic acid.

Time of L exposure	N	Perinuclear luminescence	w/ss*	Cytoplasmic microsource luminescence
CT 0000-0005	7	7/7 Stage I—diffuse Stages II-IV—well defined	3/7	7/7 Stage I—higher concentration around nucleus Stages II-IV—in bands and bridges
CT 0000-0030	23	23/23 Stages I & V—diffuse Stages II-IV—well defined	6/23	22/23 Stage I—higher concentration around nucleus Stages II-IV—in bands and bridges showing obvious reduction in the number of microsources present with the highest concentration in the nuclear region
CT 0000-0100	21	21/21 Stages I & V—diffuse Stages II-IV—well defined	4/21	18/21 Stages I & V—evenly dispersed Stages II-IV—dramatic reduction in number of microsources, sometimes only 3 or 4 per cell
CT 0200-0300	18	18/18 Stages I & V—diffuse Stages II-IV—well defined	8/18	6/18 Stages I & V—dramatic reduction in number of microsources Stages II-IV—no microsources

* With microsource-like substructure.

= 2,600, N = 56). Linear regressions of numbers of microsources per 2,500 μm^2 against cell surface area indicated no correlation between the density of microsources within a given area and cell size. However, there is significant correlation between the total number of microsources per cell and cell size (correlation coefficient for all cells stimulated with acetic acid = 0.395, N = 42, $P < 0.01$) and, therefore, comparisons between groups in Tables IV and V were made for microsources per 2,500 μm^2. Table IV lists results from the three different stimulus modes: mechanical, and formic and acetic acids. With mechanical stimulation, counts were made from the first flash (FF) alone as well as in conjunction with subsequent flashes (SFs). SFs were only counted for as long as enough microsources appeared to stay in register from one flash to the next to indicate that no cell movement had occurred.

Acetic acid was usually employed to stimulate the cells in making counts, since it is commonly used to stimulate bioluminescence in dinoflagellates and physiological evidence indicates that it is relatively harmless (Widder & Case, 1981). It also had the advantage of being less likely than mechanical stimulation to cause cell movement.

Formic acid was also tested because it induced a more rapid onset and decay of luminescence that we hoped would reduce microsource movement during the stimulus period. Image intensification analysis of formic acid-stimulated cells revealed that clumps of microsources flashed in near synchrony and that frequently a microsource flashed only once or twice, making it difficult to distinguish the number of microsources in a clump.

Two-sample *t*-test comparisons of the different stimulus modes indicated no statistical difference between counts made from the mechanically, formic acid- or acetic acid-stimulated cells. Counts made from FF and SFs, however, were statistically different from all three groups.

Microsource counts using acetic acid stimulation were also made on cells that were maintained in darkness (DD) for more than 24 h and then tested in their subjective night phase (CT 1400–1900) (Table V) after being in the holding tubes between 28 and 33 hours. These counts were compared with night-phase cells that had received a normal light-dark (LD) cycle while being maintained in a holding tube for the same period of time. Microsource counts from the two groups were not statistically different. There was also no difference between the LD cells maintained in the holding tube for 28 to 33 hours before acetic acid stimulation (Table V), and those that were loaded into the tubes only 4 to 9 h before acetic acid stimulation in the same part of the night phase (CT 1400–1900) (Table IV).

Fluorescence of bioluminescent sources

Since the bioluminescent sources in *Noctiluca* are brightly fluorescent (Eckert and Reynolds, 1967), we looked for the same phenomenon in *Pyrocystis fusiformis*. It could be very useful in identifying microsources for counting and measurements, as it was in *Noctiluca*. A number of filter combinations including broad-band excitation filters (BG 3, BG 12, UG 1, and UG 5) with barrier filters (410, 440, 460, 470, 500, 510, and 560), American Optical filter clusters 2072 and 2073, and the Zeiss filter cluster described in Materials and Methods failed to reveal fluorescent particles that visually correlated with the bioluminescent microsources without image intensification. When viewing the periphery of a night-phase cell (using the Zeiss filter cluster), uv excitation revealed large green fluorescent vesicles occasionally, but these were never correlated with bioluminescent activity. After approximately 15 sec of illumination small fluorescent sources began to brighten

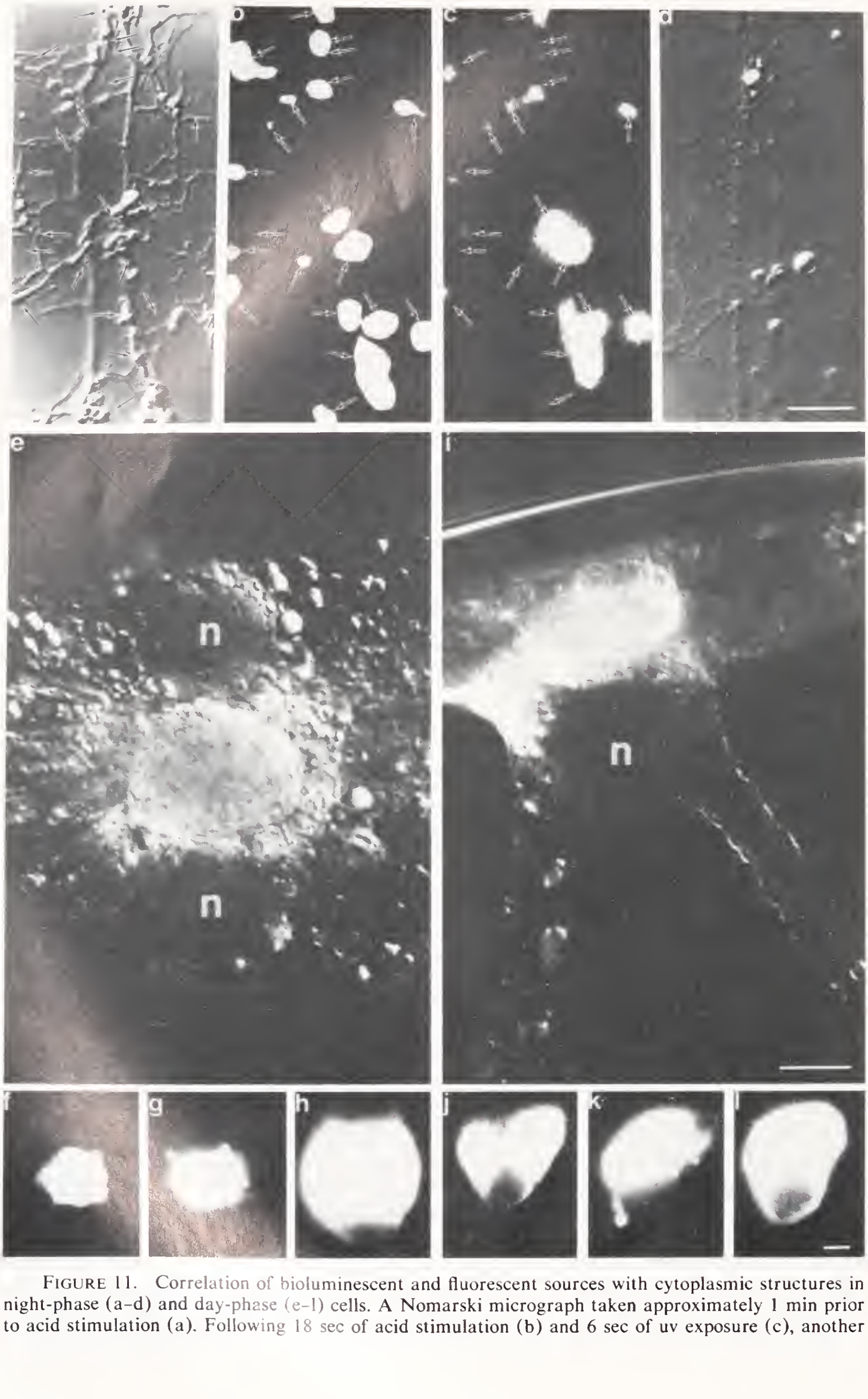

FIGURE 11. Correlation of bioluminescent and fluorescent sources with cytoplasmic structures in night-phase (a–d) and day-phase (e–l) cells. A Nomarski micrograph taken approximately 1 min prior to acid stimulation (a). Following 18 sec of acid stimulation (b) and 6 sec of uv exposure (c), another

and their appearance was associated with considerable movement and clumping, apparently due to the destructive effects of uv on the cell. After another 15 sec these fluorescent sources rapidly disappeared and after uv illumination it was generally impossible to induce bioluminescent activity from the irradiated portion of the cell.

The image intensifier revealed that the bioluminescent microsources were apparently dimly fluorescent. Night-phase cells that were stimulated to bioluminescence with either mechanical or acid stimulation and then viewed with ultraviolet illumination frequently exhibited correlations between the bioluminescent and fluorescent sources (Fig. 11b and c). When mechanical or acid stimulation occurred during the first 10 sec of irradiation, many of the fluorescent spots brightened for the time period of a normal microflash.

Nomarski micrographs were taken of the field before and after each image intensifier series. The time between recording the initial light micrograph and the first bioluminescent image of the same field recorded on video was generally about 1 minute. A typical acid stimulation sequence lasted between 15 and 20 sec and was followed by 5 to 10 sec of ultraviolet illumination. A single mechanical stimulus was used to produce a first flash. Comparison of bioluminescent sources from photographs of video recordings with corresponding loci in the Nomarski micrographs revealed no unequivocal microsource candidates. Frequently, luminescence originated from a dense clump of cytoplasmic material containing a number of different inclusions and in some instances it came from a point where no structure was apparent except for a cytoplasmic strand, or a point lacking even a cytoplasmic strand. Figure 11a–d represents one of our best series of such photographs. The most obvious microsource candidates in this and other Nomarski micrographs were the small, approximately 0.5 μm inclusions seen in many strands, although many such structures were apparently neither luminescent nor fluorescent. Other spherical or irregular inclusions larger than 0.5 μm were visible in the Nomarski micrographs, but these had no clear correspondence with the luminescent sources.

Nomarski micrographs of early day-phase cells in which the only luminescence was an acid-stimulated glow in a region near the nucleus revealed a dark yellow ovoid body (about 10 × 25 μm) surrounded by the horseshoe-shaped nucleus (Fig. 11e and i). This body has already been described and its rhythmic appearance in "day"-phase cells observed during 96 hours of continuous darkness (Sweeney, 1981). We confirm that the yellow body appears to be the source of much or all of the acid-stimulated bioluminescence and it is also strongly fluorescent (Fig. 11f–h and j–l) (Sweeney, 1980).

Nomarski micrograph was taken of the same field 1 min later (d). Arrows in (a–c) indicate equivalent loci. The fluorescent sources in (c) show good correspondence with the bioluminescent sources in (b). The brightest sources in the bottom right-hand corner of (c) were still flashing during uv exposure. In (a) the top three arrows point to the approximately 0.5 μm spherical structures mentioned in the text. In day-phase cells positioned so the nuclear region is seen in the center of the cell (e), a dark yellow spheroid is seen between the two arms of the horseshoe-shaped nucleus (n). When this cell was acid stimulated, the yellow body exhibited the round glow with substructure (f). It was also brightly fluorescent (g) and after several minutes of acid stimulation exhibited an eliptical glow with the 2 dark areas corresponding to the 2 arms of the nucleus (h). When the perinuclear region is located at one side of the cell, an optical section of the nucleus (n) shows the branch point of the 2 cytoplasmic strands, which cross the central vacuole (i). The yellow body lies between the nucleus and the cell wall. Initial response of the cell in (i) to acid stimulation (j) followed by uv excitation (k) and further acid stimulation (l) is shown. Scale, 10 μm. Scale bar in (d) applies to (a–d), in (i) applies to (e) and (i), and in (l) applies to (f–h) and (j–l).

TABLE IV

Effects of stimulus mode and cell size on numbers of microsources seen in night-phase cells.

Stimulus	Stage	MSs†/2500 μm²	cell surface area (×10⁵ μm²)	MSs/cell (×10³)
Mechanical				
FF	I	41	1.4	2.3
FF + 1 SF		45		2.5
FF	II	25	1.4	1.4
FF + 7 SFs		35		2.0
FF	III	23	1.5	1.4
FF + 3 SFs		59		3.5
FF	II	14	2.0	1.1
FF + 3 SFs		67		5.4
FF	II	24	2.2	2.1
FF + 6 SFs		75		6.6
FF mean + SD		25 ± 10		1.7 ± 0.5
FF + SFs mean ± SD		56 ± 16		4.0 ± 2.0
Formic Acid				
6 *M*	II	38	1.6	2.4
6 *M*	II	26	1.6	1.6
6 *M*	II	26	2.0	2.1
23 *M*	II	22	2.5	2.2
mean ± SD		28 ± 7		2.1 ± 0.3
Acetic acid (4 *M*)				
	I	26*	2.6	2.7
	I	31*	2.8	3.5
	III	74	3.4	10.0
	III	37*	3.5	5.2
	II	37*	3.7	5.5
	III	27*	4.0	4.3
	II	31*	4.3	5.3
	II	34*	4.5	6.1
	I	34*	4.7	6.4
	III	35*	4.8	6.7
mean ± SD		37 ± 14		5.6 ± 2.0

† MSs = microsources.

* Counts were made from 1849 μm² area and normalized to 2500 μm².

Cells maintained in holding tube less than 9 h on normal LD cycle and tested between CT 1400 and CT 1900.

DISCUSSION

The distribution of bioluminescence undergoes striking changes during the daily cycle of *Pyrocystis fusiformis.* Most apparent were the total disappearance of microsources from the periphery of early day-phase cells and the development of the perinuclear glow. While the appearance and disappearance of the perinuclear glow and the rhythm of mechanically excitable luminescence persisted in cells in DD, a light induction period was required before the microsources disappeared from the periphery. Since the perinuclear glow seemed to develop normally even when the microsources remained in the periphery, these two phenomena may not be causally linked. However, DD cells did not seem to develop the microsource-like substructure in the perinuclear glow, characteristic of some early day-phase cells. This observation, along with the apparent concentration of the microsources

TABLE V

Effect of a 24 h dark cycle (DD) on numbers of microsources seen in night-phase cells.

Stage	MSs†/2500 μm	Cell surface area ($\times 10^5$ μm^2)	MSs†/cell ($\times 10^3$)
LD			
II	33	2.7	3.6
I	24	2.7	2.6
II	11*	2.8	1.2
II	45*	3.1	5.6
I	14*	3.5	2.0
I	33*	3.6	4.8
I	53*	3.8	8.1
II	32	3.9	5.0
II	34	4.0	5.4
I	62*	4.0	9.9
II	22*	4.1	3.6
II	21	4.2	3.5
III	31*	4.6	5.7
III	24*	4.7	4.5
mean ± SD	31 ± 14		4.7 ± 2.3
DD			
II	36	2.2	3.2
I	57	2.6	5.9
I	20	2.6	2.1
II	40	2.7	4.3
III	22*	2.7	2.4
I	30*	2.9	3.5
II	36	2.9	4.2
I	31	3.0	3.7
II	50*	3.0	6.0
III	55*	3.0	6.6
I	51*	3.3	6.7
I	28	3.7	4.1
II	63	3.9	9.8
II	14	4.2	2.4
III	61*	4.4	11.0
I	26*	4.5	4.7
III	70*	4.6	13.0
II	14*	4.8	2.5
mean ± SD	39 ± 18		5.3 ± 3.1

† Microsources.

* Counts were made from 1849 μm^2 area and normalized to 2500 μm^2.

Cells in both LD and DD were maintained in holding tubes between 28 h and 33 h before testing between CT 1400 and CT 1900. Stimulus mode was addition of 0.05 cc of 4 *M* acetic acid to 1.5 cc SW bath.

in the bands and bridges of stages II–IV during the transition phases, suggested that the microsources might have been following a migration pattern opposite to that observed for the chloroplasts (Sweeney, 1981). If so, the nonstructured perinuclear glow that developed, whether or not the microsources disappeared from the periphery, might be attributed to the synthesis of bioluminescent substrates prior to their packaging in microsources.

If the microsources are normally broken down and resynthesized in the perinuclear region every day, then in DD cells, when the microsources seem to remain in the periphery, we might expect to see twice the normal density of microsources in the periphery following the synthesis and outward migration of new microsources in the following night phase. Comparison of microsource density in LD and DD cells (Table V) indicated this was not the case. Furthermore, there was no greater TMSL of DD over LD night-phase cells; DD cells actually produced less light than LD cells (Fig. 10).

The apparent spreading of the perinuclear glow seen in the transition periods at dawn and dusk might indicate that the bioluminescent substrates, rather than the microsources themselves, migrated, or perhaps both the microsources and the substrates migrated independently.

Whatever the basis for the appearance and disappearance of the microsources and the perinuclear glow, the same rhythmic changes probably occur in *Pyrocystis noctiluca*, since our preliminary observations have shown the presence of a perinuclear glow and the absence of microsources in early day-phase cells and microsources spread over the surface of night-phase cells.

It has been suggested that the decrease in mechanically excitable luminescence in day-phase cells of *Pyrocystis* and several other dinoflagellates is due to an increase in the threshold for mechanical stimulation (Hamman & Seliger, 1972; Hamman *et al.*, 1981). Yet, we have recorded action potentials from both day- and night-phase cells in response to mechanical stimulation (Widder & Case, 1980). The action potential triggers a bioluminescent flash in night-phase cells, but a flash does not follow a similar mechanically inducible action potential in day-phase cells. Our present findings indicate that the lack of mechanically excitable luminescence of day-phase cells is not due to an absence of microsources, because acid stimulation revealed microsources in late day-phase cells as well as in the early "day" phase of cells in DD. Therefore, a link between the mechanically stimulated action potential and the microsource in night-phase cells is absent or refractory in day-phase cells. Since acid stimulation circumvents mechanical inexcitability in day-phase cells, it may be that protons are required to trigger bioluminescent activity, as suggested previously (Hastings, 1978). Therefore, the rhythm of mechanically stimulable luminescence in *Pyrocystis fusiformis* might be due to rhythmic changes in proton concentrations or in membrane proton gates within the cell. Plants that perform Crassulacean acid metabolism (CAM) exhibit cyclic changes in the level of malic acid concentration found in the vacuole (Lüttge and Higinbotham, 1979). Acid concentration reaches a peak towards the end of the night phase and decreases by an order of magnitude at the end of the day phase. If similar changes occur in *P. fusiformis*, it would offer a possible explanation for a number of our observations. Besides accounting for the inexcitability of day-phase cells compared with night-phase cells, changing proton concentration would also explain the sequence of events during the transition phases. At dawn a decrease in TMSL occurs, while the number of flashes a cell can produce remains fairly constant. This is a clear indication that the cells remain mechanically excitable as their luminescent output diminishes. At dusk, when mechanical stimulation begins to evoke dim luminescence, acid stimulation reveals the presence of many brilliantly luminescent microsources. Therefore, the reduced luminescence of the transition phases is not due to a decreased mechanical excitability or an absence of luminescent substrates.

Apparently microsource number varies with cell size, while microsource density is more constant. Despite the difficulties caused by the apparent clumping of microsources and the difficulty of counting asynchronously active microsources, counts made using three different methods of stimulation (acetic acid, formic acid, and the mechanically stimulated FF) were not significantly different. Possibly the higher microsource numbers observed when SFs were included may have been due to greater movement of the microsources. The only other apparent explanation is that multiple mechanical stimuli more effectively reveal microsources in the field. Presently we do not see why that should be true.

Microsources in *P. fusiformis* were only dimly fluorescent with the filter combinations employed and only visible with the aid of the IIT. Their fluorescence was established by observing a field of bioluminescent sources corresponding to fluorescent sources at the same loci. These fluorescent sources were often seen to brighten briefly for the duration of a microflash when mechanical or acid stimulation was applied during the first few seconds of uv irradiation. The best focus for the fluorescent sources also corresponded to the best focus for the bioluminescent sources.

Since microsource identification was only possible with the IIT, attempts at correlating luminescent sources with cytoplasmic structures required a time delay of one minute or more. The possibility that cytoplasmic streaming could displace microsources during this delay complicated the comparisons. In general, luminescence seemed to originate from the junctions of cytoplasmic strands where several different cytoplasmic inclusions were clumped together. In cases where no cytoplasmic structure was seen at the location of a luminescent source we must assume either that the source was smaller than the resolving power of the light microscope, or that some movement had occurred. Our observations suggest that the microsources are 0.5 μm or less in diameter.

ACKNOWLEDGMENTS

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STUDIES ON REPRODUCTION IN THE HERMAPHRODITIC SEA STAR, *ASTERINA MINOR*: THE FUNCTIONAL MALE GONADS, "OVOTESTES."

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ABSTRACT

Development of gonads in the sea star, *Asterina minor* Hayashi was studied by light microscopy. In adults, two series of gonads are found in one interradial area. Each series consists of an ovary, supernumerary ovaries, and ovotestes with a regular alignment to the margin. In breeding season, the ovotestes have sperm and some small basophilic oocytes. After releasing sperm, the ovotestes are much reduced in size, but the small oocytes remain. After spawning, as no rapid increase of testicular tissue occurs, these gonads resemble supernumerary ovaries. In addition to the previously existing series of gonads, a few new ovotestes are formed in more marginal positions annually. During and after the spawning period, the germinal cells in the genital rachis increase in number, differentiate into ovotestes, and form a gonoduct and gonopore of their own.

Hermaphrodism in this species is maintained by the annual formation and maturation of ovotestes and by the annual maturation of oocytes in ovaries (*cf.* Fig. 1).

INTRODUCTION

Most species in the genus *Asterina* are dioecious. However, hermaphroditic species have also been reported among *Asterina: Asterina scobinata* (Dartnall, 1970), and occasional hermaphrodites in *Asterina batheri* (Ohshima, 1929). In *Asterina gibbosa* sexual change occurs from male to female (Cuénot, 1898; Bacci, 1951; Delavault, 1966). These studies suggest the diversity related to sex in *Asterina.*

In the small sea star, *Asterina minor* (Hayashi, 1974), Komatsu *et al.* (1979) reported that hermaphrodism is the normal sexual condition and that the gonads consist of both ovarian and testicular tubules. However, the annual reproductive cycle is not yet known.

In the present study the morphology and histology of ovotestes including testicular tissue were examined in *Asterina minor* from Tokyo Bay through two reproductive cycles.

MATERIALS AND METHODS

Specimens of *Asterina minor* (10 animals on the average) were collected approximately monthly for two years, from April 1979 to March 1981, from the intertidal zone of Hashirimizu, Kanagawa Prefecture, Japan. Animals ranged from

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Abbreviation: R, major radius (distance from the center of the disk to the tip of an arm).

TABLE I

The relationship between the size of animal (R) and the condition of the reproductive organs in Asterina minor *in April and May (1979, 1980).*

R* (mm)	Number of animals	Ovaries	Supernumerary ovaries	Ovotestes	Number of gonopores in each series of gonads
2.0–2.9	9	+	–	–	0
3.0–3.9	19	+	–	–	0–1
4.0–4.9	11	+	–	–	1
5.0–5.9	11	+ (M)	–	+ (M)	2–3
6.0–6.9	4	+ (M)	–	+ (M)	3–4
7.0–7.9	6	+ (M)	– or +	+ (M)	3–6
8.0–11.5	8	+ (M)	+	+ (M)	4–9

Presence of gonads and maturation were designated as follows: –, absent; +, present; M, mature sexual cells.

*R = major radius.

2.3 mm to 11.5 mm in major radius, the distance from the center of the disk to the tip of an arm.

The animals were fixed with 10 per cent formalin in sea water for a week, followed by decalcification with 10 per cent trichloroacetic acid for another week at 4°C. After dehydration in a graded ethanol series and embedding in paraffin, the whole bodies of the animals were sectioned serially 5 to 7 μm thick. Horizontal and transverse sections were stained with hematoxylin and eosin.

RESULTS

Three types of gonads are present in *Asterina minor*: ovary, supernumerary ovary, and ovotestis. The ovary is the largest gonad and contains oocytes all year round. Supernumerary ovaries are much smaller than the ovary and always contain young or mature oocytes. The ovotestes, the main target of this paper, are hermaphroditic gonads having testicular tissue and oocytes or, in a small number of cases, testicular tissue only. The three types of gonads align to form a series, and two series are found in each interradial region.

Correlations between body size and gonads

During April and May (the breeding season of *Asterina minor* in Tokyo Bay) when the gonads are sharply distinguishable, the maturity of gonads was compared with the measurements of the body size, namely major radius (R) in Table I. Almost all individuals larger than 5 mm in R are hermaphrodites whose gonads consist of ovotestes and ovaries; sperm and young oocytes in the former, large eosinophilic maturing oocytes in the latter. For each series of gonads, two or more gonopores are found in the oral side of the disk.

Individuals larger than 5 mm are adults with mature oocytes and sperm. Most individuals under 5 mm in R are immature sea stars having only one immature ovary for each series, and individuals 3.5–5 mm in R have one gonopore.

Three types of gonads in breeding season

The centrally located ovary expands into one or more branched tufts protruding into the coelomic cavity (Figs. 1, 2A, B). A gonoduct connects with the orifice of

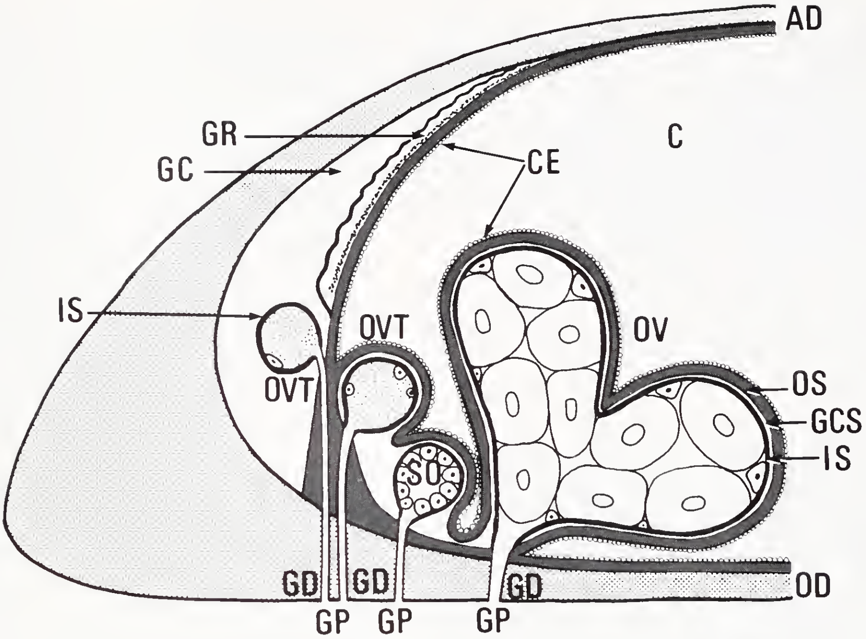

FIGURE 1. Diagrammatic representation of the gonad's alignment in a series of gonads during the breeding season of *Asterina minor*. The three types of gonads, ovary (OV), supernumerary ovary (SO), ovotestis (OVT) are arranged regularly from central (right) to marginal and oral (below) to aboral. The relationship of the gonads to the genital coelom are shown. AD, aboral side of the disk; C, coelomic cavity; CE, coelomic epithelium; GC, genital coelom; GCS, genital coelomic sinus; GD, gonoduct; GP, gonopore; GR, genital rachis; IS, inner sac; OD, oral side of the disk; OS, outer sac; OV, ovary; OVT, ovotestis; SO, supernumerary ovary.

the ovary and penetrates the body wall and opens in the gonopore. The oocytes reach more than 400 μm in diameter and have a large quantity of eosinophilic yolk. Small basophilic oocytes also occur in the spaces between the large eosinophilic oocytes (Fig. 2E). These two stages of oocytes are surrounded by follicle cells and are attached to the basal germinal membrane. Infrequently, gonial cells are also observed in the basal germinal membrane. The ovarian wall is composed of the inner and outer sacs, the interspace being the genital coelomic sinus, as described by Walker (1974) (Figs. 1, 2C, D). The outer sac consists of connective tissue and muscle, and is covered by a single layer of coelomic epithelium. The inner layer of the inner sac is the germinal epithelium.

A few ovotestes align at the most marginal and aboral region (Fig. 1, 2A, B). They are smaller than the ovary, but have independent gonoducts. The ovotestes form fully developed spermatogenic columns with spermatogonia, spermatocytes and spermatids, and sperm accumulating in the lumen of the gonads (Fig. 2F). In addition to these spermatogenic cells, small basophilic oocytes are frequently found in the ovotestes (Figs. 2F, G, 4F). The small oocytes are in contact with the basal germinal membrane and are enclosed by follicle cells. The number and the size of the small oocytes vary in different ovotestes within the individual as well as among different individuals, but the oocytes do not accumulate enough yolk to reach the maximum size. The ovotestes are of two types determined by their position: one type protruding into the coelomic cavity, surrounded by the inner and the outer sac (Fig. 1, right ovotestis, 2D, left ovotestis); the other type remaining in the

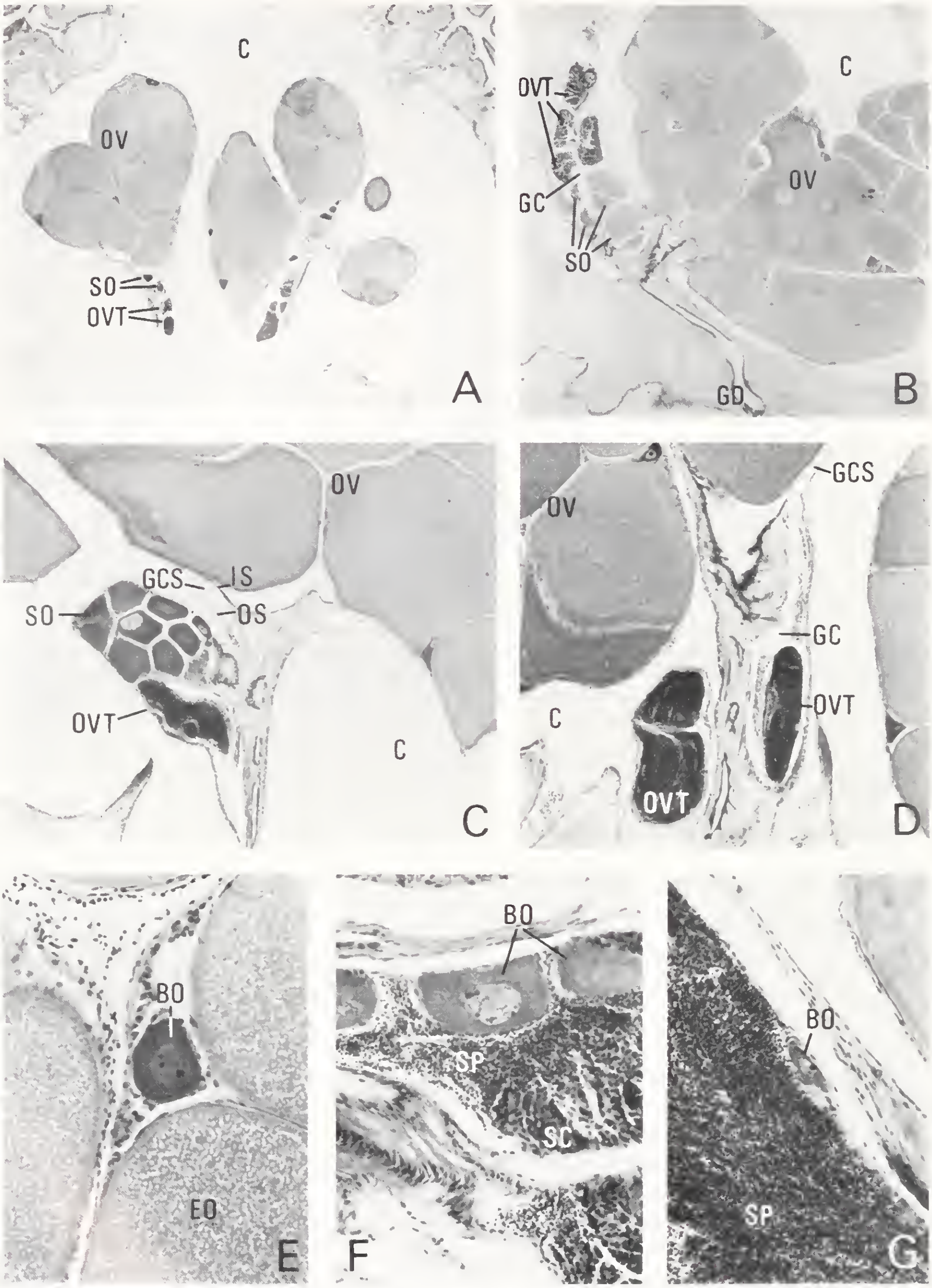
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FIGURE 2. The alignment of the three types of gonads and their sexual cells.

2A. (May) Two series of gonads. Positions of the gonads are from central (upper) to marginal. The left series of gonads has an ovary (OV), two supernumerary ovaries (SO), and two ovotestes (OVT). ×25.

2B. (January) A growing ovary, four supernumerary ovaries, and three ovotestes are shown. The latter are in the genital coelom (GC). The gonoducts (GD) of all the gonads extend separately but are not shown in this section. ×25 (transverse section, others are horizontal).

2C. (May) A supernumerary ovary having small basophilic oocytes is lying between the mature genital coelom, surrounded only by the inner sac (Fig. 1, left ovotestis, 2D, right ovotestis).

A variable number of the supernumerary ovaries with gonoducts occur between the ovary and the ovotestes (Figs. 1, 2A, B, C). Larger animals tend to have more than one supernumerary ovary (Table I). The supernumerary ovaries have fewer oocytes than the ovary, and infrequently have oocytes with yolk accumulated.

In each series of gonads, the ovary, the supernumerary ovaries, and the ovotestes align regularly from central to marginal and oral to aboral (Figs. 1, 2A, B).

Spawning

In Tokyo Bay, spawning of *Asterina minor* takes place in May. After spawning, the ovaries decrease in size, but small basophilic oocytes remain and non-germinal cells increase greatly in number (Fig. 3A).

By the time the ovotestes are ready to spawn, the gonads are filled with sperm (Fig. 2G). Following sperm release, the ovotestes reduce greatly in size, and the remaining small basophilic oocytes come into close proximity to each other (Fig. 3B). After spawning, the remaining sperm are gradually phagocytized by non-germinal cells and the spawned ovotestes look like supernumerary ovaries (Figs. 3C, D).

Formation of new ovotestes

During the breeding season or after spawning time, new gonads are formed. From the aboral haemal ring, two genital rachises extend downward in each interradial area (Figs. 1, 4A, B). Therefore, the genital rachis is more aborally positioned than the series of gonads. The germinal cells in the rachis begin to increase in number and swell out into the genital coelom (Figs. 3B, 4C, D). The thin layered, small gonads do not develop gonoducts nor oocytes but remain as lumps of germinal cells. However, thick layered, large gonads which are obviously ovotestes, acquire gonoducts and small basophilic oocytes (Fig. 4E). In November, the most developed gonads differentiate spermatogenic columns and sperm, and the enlargement of the gonads continues until the next breeding season (Figs. 4F, G).

In adults, a few new ovotestes are formed every year in the marginal and aboral position of each series of gonads. Their development differs within an individual as well as among different individuals.

DISCUSSION

Gonads are formed from the genital rachis in echinoderms. Houk *et al.* (1980) studied the genital rachis in juvenile sea urchins and its gonad formation. In adult

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ovary and the ovotestis. In the ovary two sacs of gonadal wall, inner sac (IS) and outer sac (OS), with the intervening genital coelomic sinus (GCS) are shown. ×62.5.

2D. (May) A large mature ovary and the mature ovotestes are shown. The left ovotestis is surrounded by two sacs and the right one in the genital coelom is surrounded by the inner sac only. ×62.5 (compare with Fig. 1).

2E. (May) An ovary having large eosinophilic oocytes (EO) and small basophilic oocytes (BO). ×250.

2F. (April) Ovotestes with spermatogenic columns (SC), sperm (SP), and some small oocytes. The oocytes are surrounded by follicle cells. ×250.

2G. (May) The ovotestis filled with mature sperm and a small basophilic oocyte that is in contact with the basal germinal membrane. ×250.

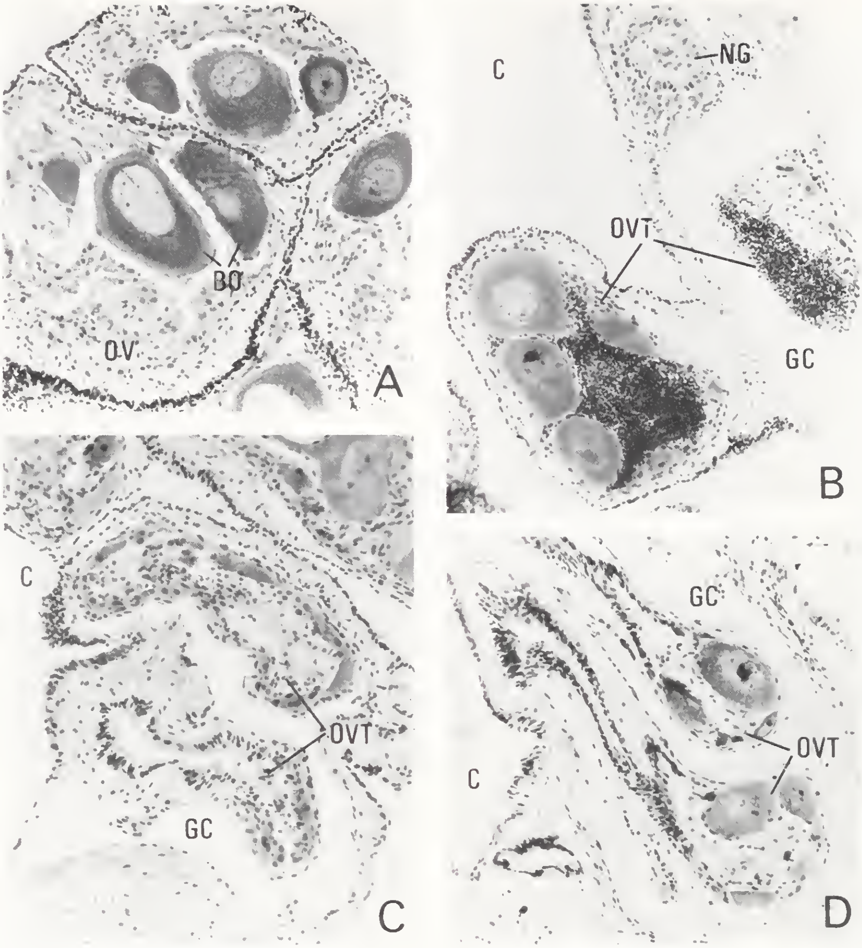

FIGURE 3. The spawned gonads.

3A. (June) Spawned and reduced ovary (OV) having small basophilic oocytes (BO) and a large number of non-germinal cells. ×250.

3B. (July) Ovotestes (OVT) releasing sperm. The oral (lower left) ovotestis having several small oocytes is surrounded by two sacs and projects into the coelomic cavity (C). The right ovotestis in the genital coelom (GC) having fewer oocytes is surrounded only by the inner sac. The aboral cell mass is a new gonad (NG). ×250 (transverse section, others are horizontal).

3C. (August) Two marginal and aboral ovotestes in the genital coelom. The upper ovotestis contains a few sperm which are being phagocytized by the non-germinal cells. ×250.

3D. (August) Two marginal and spawned ovotestes look like supernumerary ovaries. ×250.

sea urchins the rachis disappears. Adult ophiuroids have the genital rachis in the aboral haemal ring and the branches of the rachis form the gonads (Hyman, 1955). In the juvenile *Asterias rubens*, the genital rachis is formed in the aboral haemal ring with its 10 branches and establishes the gonads (Gemmill, 1914). On the contrary, in *Asterina minor*, the genital rachis remains until adult and forms ovotestes annually.

It is probable that the spawned ovotestes change into the supernumerary ovaries. During and after the breeding season, since there are very few gonial cells in most ovotestes, it is not conceivable that a large quantity of testicular tissue differentiates once more in the spawned ovotestes. Moreover, the oocytes in the ovotestes do not

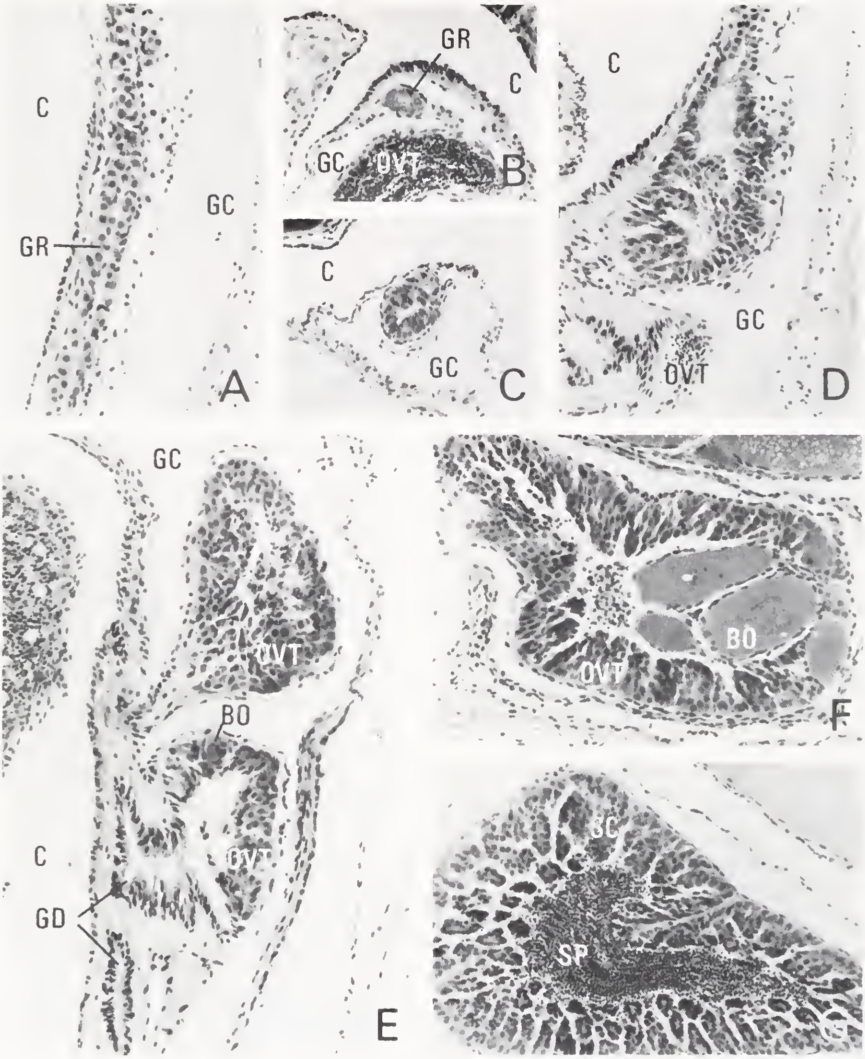

FIGURE 4. Formation of new gonads. A, D, E, and G are transverse and B, C, and F are horizontal.

4A. (May) A longitudinal section of the genital rachis (GR) which is adjacent to the wall of the genital coelom (GC). ×250.

4B. (May) The tubes of the germinal cells in the genital rachis are shown. The ovotestis (OVT) in the genital coelom is filled with sperm. ×250.

4C. (August) The germinal cells increase in number and swell out into the genital coelom. The new gonad has not formed its gonoduct yet. ×250.

4D. (July) The swollen part of the genital rachis. The oral (below) spawned ovotestis has a small number of relict sperm. ×250.

4E. (September) Two ovotestes having their own gonoducts (GD). A small basophilic oocyte (BO) is shown in the lower ovotestis. ×250.

4F. (November) An ovotestis having testicular tissue and small oocytes. Most of the oocytes are in contact with the basal germinal membrane. ×250.

4G. (January) An ovotestis having spermatogenic columns (SC) and sperm (SP). ×250.

mature. In the ovary, there are two stages of oocytes in the breeding season: maturing eosinophilic oocytes and small basophilic oocytes. Unpublished data show that the basophilic oocytes may be the source of the eosinophilic oocytes of the next breeding season. If the spawned ovotestes underwent testicular tissue differentiation some time out of season, second year oocytes should be formed together with mature sperm in once spawned ovotestes. Examples in which about two years are required for oocyte development have been reported by Worley *et al.* (1977) and Chia (1968).

The observations suggest that in *Asterina minor*, the ovotestes are protandric, are formed annually, and after spawning may change into the supernumerary ovaries. The hermaphrodism of this species is maintained by the ovotestes and the ovaries.

Acknowledgments

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ONTOGENY OF PHOTOTAXIS DURING LARVAL DEVELOPMENT OF THE SEDENTARY POLYCHAETE, *SERPULA VERMICULARIS* (L.)

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ABSTRACT

Responses of *Serpula vermicularis* larvae to light were studied in the laboratory during all stages from hatching to settlement. Early trochophores swam upward but were indifferent to light until development of an eyespot at three days. After this, and for most of larval life (about 25 days), dark-adapted larvae initially displayed a positive phototaxis when exposed to horizontally directed light. After a period of adaptation, they changed the sign of their response to negative, the time required for the onset of this change being an inverse function of intensity. Trochophores responded to all wavelengths of light from 350 nm to 625 nm, but were most sensitive to those below 525 nm. Metatrochophores, which had two eyespots, were continuously and strongly photonegative. Benthic nectochaete larvae were indifferent to light while crawling on the bottom, but at metamorphosis they generally oriented the primary tube aperture away from the light. Based on laboratory behavior, a twilight migration pattern is predicted for *S. vermicularis* trochophores, and a hypothesis is suggested which could account for the evolution of diel migration in larvae of coastal species.

INTRODUCTION

Quantitative data on responses of invertebrate larvae to environmental stimuli help us understand the often complex interaction of factors influencing larval distribution, and also enable us to predict larval movements for species which are rarely encountered or difficult to sample in the field. Although general patterns of photoresponse have been described for at least some larvae in virtually every phylum (Thorson, 1964), the importance of light intensity, wavelength, and light or dark adaptation remain poorly understood for most common larval forms. For the most part, quantitative studies have been limited to economically important species such as cirripedes (Lang *et al.*, 1979), bivalves (Bayne, 1964) and brachyurans (Forward and Costlow, 1974; Ritz, 1972a), and species with short-lived lecithotrophic larvae such as bryozoans (Ryland, 1960) and compound ascidians (Crisp and Ghobashy, 1973).

In this study, we quantified the ontogenetic changes in photoresponse of lab-reared *Serpula vermicularis*, a species with extended planktotrophic development, with the objective of understanding how behavior might influence vertical distribution of the larvae and, ultimately, recruitment. In the San Juan Islands, Washington state, where our work was carried out, low larval abundance and complex tidal currents make it virtually impossible to study larval distribution by conventional sampling techniques.

S. vermicularis is a common epifaunal species occurring on hard substrata in boreal, temperate and tropical seas throughout the world (Ushakov, 1965). Al-

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though larval behavior of *S. vermicularis* has not been investigated, larvae of related serpulids show several different patterns of photoresponse. For example, *Pomatoceros triqueter* remains photopositive throughout larval life (Segrove, 1941), while *Hydroides norvegica* is indifferent to light at first, only slightly photopositive later, and at settlement may be either photopositive or photonegative (Wisely, 1958).

MATERIALS AND METHODS

Larval culture

Adult specimens were collected from April to August, 1979 to 1981, from the rocky intertidal zone near Pt. Caution, San Juan Island, Washington. Spawning was induced by removing the animals from their tubes. This was accomplished without damaging the animals by carefully breaking the ends of the tubes and blowing the worms out with compressed air, posterior end first (R. L. Fernald, personal communication). Worms were isolated in bowls of seawater where ripe ones generally began spawning within 10 minutes.

Coelomic fluid and immature oocytes were removed by decanting off the seawater after the eggs had settled. The eggs were washed several times in filtered seawater, then fertilized with a small amount of dilute sperm suspension. Excess sperm were removed by rinsing the eggs several more times. Cultures were maintained in 8 liter jars of filtered seawater, stirred continuously by paddles rotating at 60 rpm, and kept cool in running seawater aquaria. After hatching, trochophores were fed daily with the green flagellate, *Dunaliella tertiolacta*. Culture water was changed every three days until the larvae became benthic, after which time only weekly changes were made, since larvae were lost easily during this stage. Overhead fluorescent lights were on for an average of three hours each evening. During the daytime, most light came through large laboratory windows; light intensity in the cultures thus fluctuated in a natural manner. On a typical day, the cultures experienced intensities comparable to those between 5 and 10 m depths in the field (Young and Chia, 1982).

Whenever a larva metamorphosed in culture, we recorded the orientation of its tube relative to the window light, which was predominantly unidirectional.

General experimental techniques

All experiments were carried out at night in a darkened room. The experimental chamber consisted of a rectangular aquarium constructed of microscope slide glass, and measuring 7.5 cm long, 2.5 cm wide, and 2.5 cm deep. The chamber was filled to a depth of 1.0 cm and placed in a wooden box 25 cm long, 10 cm wide, and 10 cm deep, which was painted with mat black paint on all internal surfaces in order to minimize reflections. The lid was lined with black felt to form a light-tight seal. Light was projected through a 3 cm diameter hole in one end of the box, and the intensity was measured with a Li-cor quantum meter (Lambda Instrument Company, Model LI-185) through a similar hole in the opposite end. The light meter was sensitive primarily in the range of the human visual spectrum (400–700 nm) and could detect light at intensities as low as 0.1 $\mu E \cdot m^{-2} \cdot sec^{-1}$. During experiments with white light ("intensity experiments") the larvae were observed with the lid of the box removed, while in "wavelength experiments" the lid was left on the box until the experiment was terminated.

The light source was a Bausch and Lomb projecting lamp with a General Electric "CPR" incandescent bulb. Although the projector was fitted with a rheostat, we always used it at the highest setting and changed light intensity by stopping down the aperture, imposing neutral density filters, or altering the distance between the lamp and the experimental chamber. In this way, changing the spectral characteristics of the emitted light was avoided.

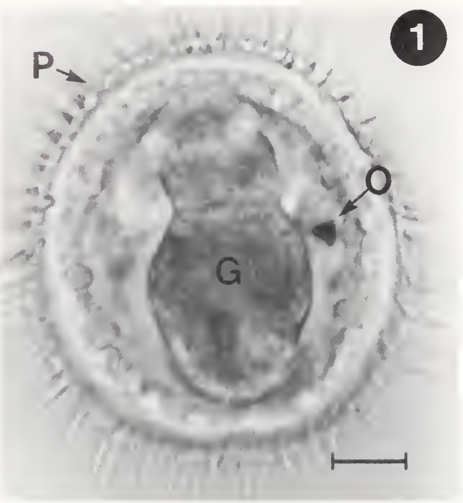
Wavelength experiments

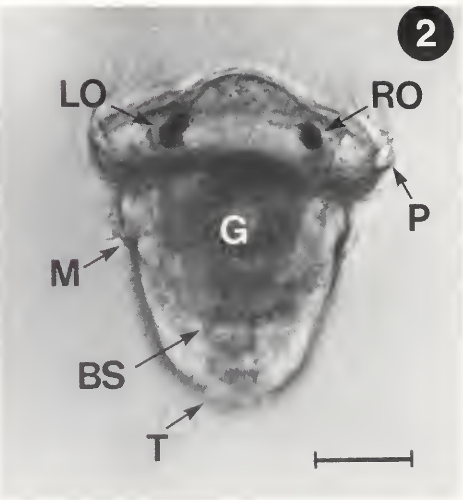
Colored light of discrete wavelengths (10 nm bandwidth) was produced by inserting a Bausch and Lomb diffraction grating monochromator in the projector beam. Ideally, action spectra for behavioral phenomena are derived from "equal response" curves (Forward and Cronin, 1979), in which a particular behavioral level is plotted on a graph of wavelength *vs* energy. However, since our light meter was relatively insensitive in the infrared and ultraviolet regions, and emitted only very low energy levels near the lower end of the spectrum, we found it impossible to characterize responses at multiple energy levels. Instead, we used the maximum intensity obtainable below 425 nm and held intensity constant at 1.0 $\mu E \cdot m^{-2} \cdot sec^{-1}$ over the rest of the spectrum, then plotted the degree of the response against wavelength. Although data obtained in this way are not as useful from a physiological standpoint, they do indicate the colors of light which the larvae can sense, and are thus useful for ecological inference.

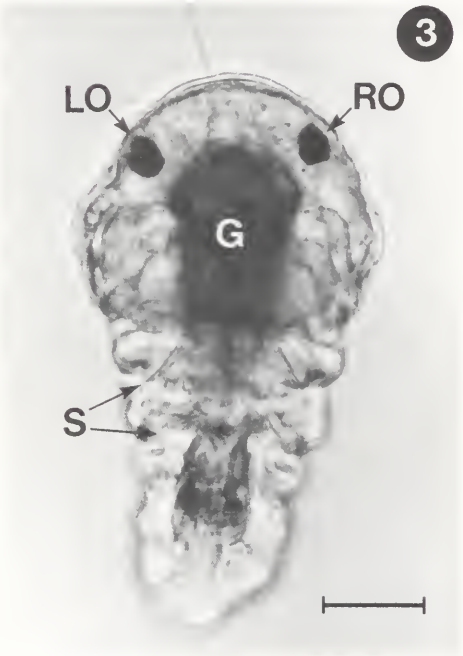
The experimental protocol was as follows. Several hundred larvae, dark adapted for at least 10 min, were pipetted into the glass experimental chamber in the dark, and mixed thoroughly by drawing water in and out of the pipette several times. Monochromatic light was shone through the chamber horizontally for 5 min, then extinguished. Samples were then taken immediately and simultaneously from the two ends and the middle of the chamber. The sampling apparatus consisted of three 50 ml hypodermic syringes, fitted with 18 ga needles and held securely in a plexiglass frame. A strip of plexiglass attached to the plungers was lifted quickly in order to withdraw 30 ml samples from all regions at the same time. The samples were discharged into Bogorov plankton counting trays and counted under a dissecting microscope. A fresh group of larvae was used for each wavelength tested.

Intensity experiments

In experiments using white light, we wished to consider the effect of light adaptation by noting changes in larval distribution over time. Since this required that we sample the experimental chamber repeatedly during each run, we needed a sampling procedure which did not remove larvae from the chamber. Initially, we attempted photographing the distributions with high speed film. Although the larvae were easily resolved in the photographs, this method proved unsatisfactory because larvae swarming against the ends of the containers were impossible to count. To avoid this problem, we counted the animals visually through five 2 mm wide slits in a black card resting on top of the chamber. One slit was above each end and the other three were equally spaced. All slits were aligned perpendicular to the light source. At any given sampling time, we began by counting the larvae visible through the slit nearest the light, then proceeded to the opposite end, counting through each slit in turn. The entire procedure took between 45 and 60 seconds, and was carried out at two minute intervals. Larvae from a single culture were used for each experiment. Two subsamples of the culture, containing roughly equal numbers, were placed in separate chambers. These were used for alternate intensity levels in order to expedite the data collection; while one chamber was being exposed







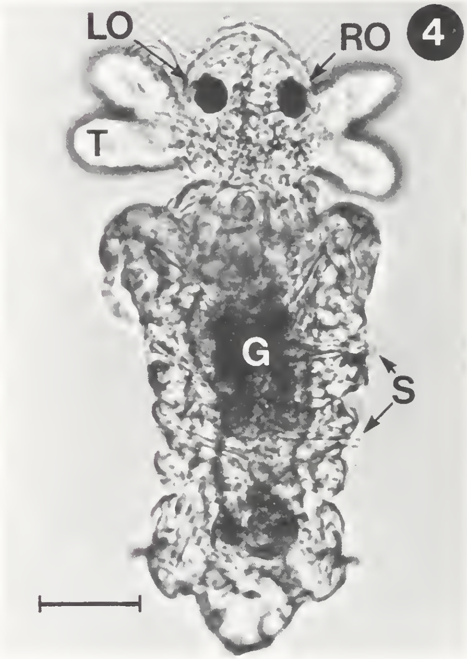


FIGURE 1. Anterior view of 3 day old *Serpula vermicularis* trochophore, showing newly developed right ocellus (O), gut (G), prototroch (P). Scale bar equals 10 μm.

FIGURE 2. Twenty-six day old *S. vermicularis* metatrochophore viewed dorsally, showing right ocellus (RO), left ocellus (LO), prototroch (P), metatroch (M), telotroch (T) and body segments (BS). Scale bar equals 50 μm.

FIGURE 3. Thirty day old *S. vermicularis* benthic nectochaete larva, viewed dorsally. Setae (S) are beginning to form; other labels as in Figure 2. Scale bar equals 50 μm.

FIGURE 4. Fifty day old *S. vermicularis* just after the onset of metamorphosis, viewed dorsally and showing newly formed tentacle buds (T). Other labels as in previous figures. Scale bar equals 50 μm.

to light of a given intensity, the other was being cooled and dark adapted in the seawater tables. At some intensities, we repeated the experiment several times with the same group of larvae in order to assure that the general results were repeatable. However, time constraints and limited supplies of larvae prevented us from replicating quantitative trials with animals of any given age.

Photoresponse was tested at seven different intensities ranging from 10 $\mu E \cdot m^{-2} \cdot sec^{-1}$ to 1450 $\mu E \cdot m^{-2} \cdot sec^{-1}$. This range of intensities is realistic in terms of light levels the larvae might encounter during their pelagic phase. Midday light intensities measured on an overcast day in June of 1981 ranged from 1200 $\mu E \cdot m^{-2} \cdot sec^{-1}$ at the surface to less than 0.5 $\mu E \cdot m^{-2} \cdot sec^{-1}$ at a depth of 38 m.

RESULTS

Larval development

While early embryonic stages of *Serpula vermicularis* were nearly synchronous, there was considerable temporal variability within and among cultures in later development. Furthermore, the size distribution of larvae within a given dish generally became distinctly bimodal after about two weeks of feeding. Larger animals grew more rapidly and underwent more frequent morphological changes than the smaller ones; the latter never survived to metamorphosis. It is not known whether larvae of the two sizes came from the same or different females, since cultures were of mixed origin. However, it seems unlikely that the bimodality could result from competition for food, since food was always present in excess. The timing of larval development described below is based on the larger larvae, which were the only ones used for behavioral experiments. Figures 1–4 show the major features of larval development and also depict four of the five stages in which we quantified photoresponse.

The embryos hatch as trochophores one day after fertilization, and the trochophores swim for two more days before developing an ocellus. As in other serpulids (Zeleny, 1905; Segrove, 1941; Wisely, 1958), the right ocellus develops first, and is situated on the episphere, slightly nearer the prototroch than the apical organ (Fig. 1). Unlike the ocellus of *Pomatoceros triqueter*, which is black (Segrove, 1941), the photoreceptor of *S. vermicularis* is brilliant red in color. Except for some slight changes in shape (Figs. 2, 3), the ocellus remains essentially the same at the light microscope level during the entire trochophore period. Formation of the left eyespot, which occurs early in the metatrochophore stage (about 20–27 days), coincides with the onset of segmentation (Fig. 2). Both eyespots are retained at least through metamorphosis. Soon after developing the left eye, larvae enter the nectochaeta stage, during which they drop to the bottom and adopt a benthic mode of life (Fig. 3). This stage, which may begin as early as 28 days, is of variable length; some worms remain nectochaetes until at least the 50th day. Although capable of swimming, nectochaetes spend most of their time crawling on the bottom. Just before secreting the primary tube, tentacle buds appear at the sides of the head (Fig. 4). Metamorphosis, which occurs at the 5 setiger stage, took place between 41 and 50 days in our cultures.

Photoresponses of trochophores and metatrochophores

Although early larvae swam actively and swarmed in large numbers at the surface, they demonstrated no response to directional light at any intensity. They became strongly photosensitive as soon as the eyespot appeared. We subjected dark-

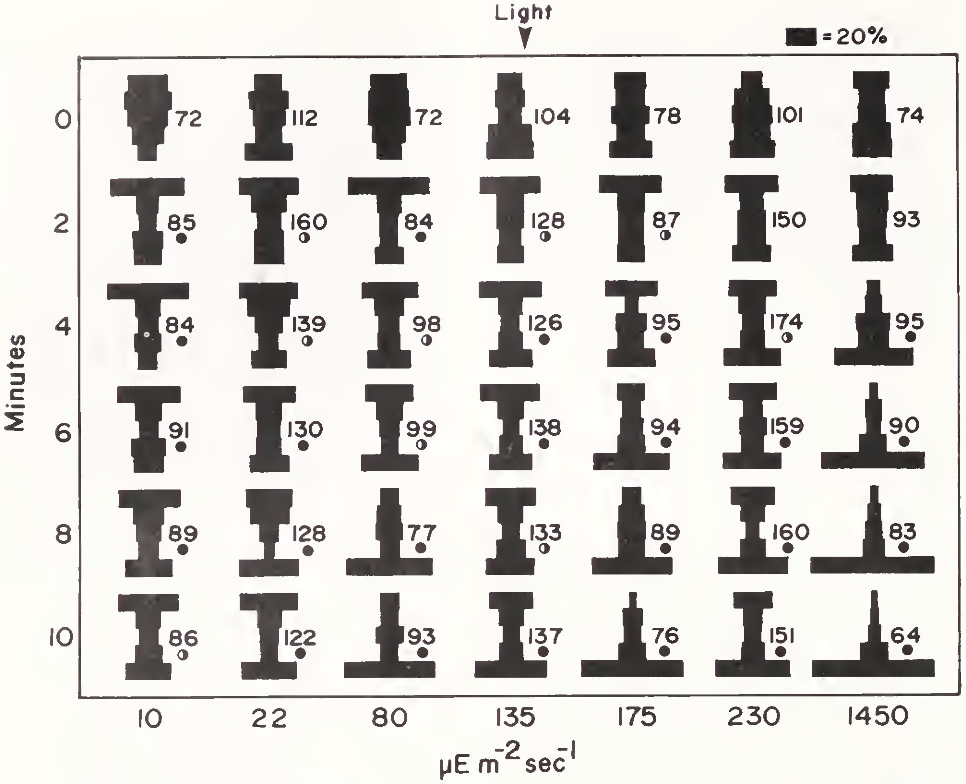


FIGURE 5. Distributions of 17 day old *S. vermicularis* trochophores exposed to different intensities of light and following various periods of light adaptation. Histogram bars show the percentages of larvae in five regions of the experimental container. Each column of graphs represents a single experiment, monitored at progressive time intervals, so the effect of light adaptation can be seen by noting changes in distribution while reading down a column. Number of larvae sampled is indicated to the right of each graph together with a symbol showing significance level, as determined by Chi-Square test for goodness of fit to a uniform distribution. Open circle: $P < 0.05$. Half-closed circle: $P < 0.01$. Closed circle: $P < 0.001$. No symbol: not significant.

adapted one-eyed trochophores to horizonal light at approximately two-day intervals. Multiple light levels were used at 6, 11, and 17 days; qualitative observations were made on the other days at a single intensity. Since the basic response did not change throughout this period, we present data from a single typical experiment (Fig. 5). Following dark adaptation, larvae responded to light of low intensity by swimming toward it. After swarming against the light side of the chamber for several minutes, they were invariably light adapted and changed the sign of their response. The time required for light adaptation was an inverse function of intensity. Indeed, at very high intensities, no photopositive period was apparent at all, though this may be because the change from positive to negative phototaxis occurred faster than the time lag inherent in our counting procedure. The overall strength of the response, as measured by the percentage of larvae responding, was also influenced by intensity. At 10 $\mu E \cdot m^{-2} \cdot sec^{-1}$, only about 40% of the larvae occupied the darkest region after 10 minutes of exposure to light, while at the highest intensity, over 70% responded. It is apparent from the graph that two different samples of

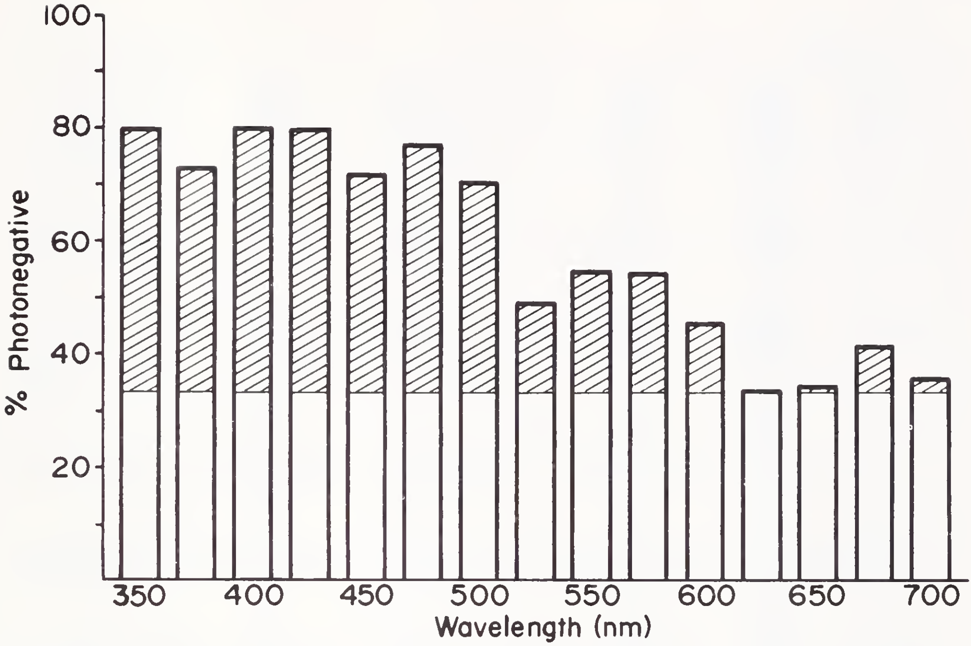


FIGURE 6. Mean (n = 2) percentages of *S. vermicularis* trochophores (4 days old) at the dark end of the experimental chamber following five minute exposure to monochromatic light of various wavelengths. Shaded segment of each bar is that portion exceeding the expected value of 33%.

larvae were used for alternate intensity levels, as the overall strength of the response differed slightly between samples. Nevertheless, the same basic trend was demonstrated by both, and the results were repeatable at a given intensity level.

Although the action spectrum for phototaxis in *Serpula vermicularis* shows a slight mode between 500 and 600 nm (Fig. 6), the highest peak is very broad, spanning all wavelengths from 350 nm (ultraviolet) to 500 nm (green). As expected, wavelengths longer than 600 nm, which penetrate to only shallow depths in the sea, were the only ones to which larvae did not respond.

With the development of the left eye, metatrochophores retained their overall negative phototaxis, but lost the initial photopositive phase of the response (Fig. 7). Phototaxis was very strong at all intensity levels tested.

Nectochaete photoresponses

After the setigerous larvae went to the bottom, they showed no response to light at any level of intensity (Fig. 8). The main behavior displayed by larvae during the creeping stage appeared to have the dual function of substratum exploration and feeding. Larvae crept about on the bottom, moving the head slowly from side to side, and apparently tasting the bottom with the mouth. Occasionally, they ingested small benthic algae.

Of several hundred larvae which survived to the nectochaete stage in our cultures, only 11 underwent metamorphosis. These larvae did not seek out and settle in the darker regions of the dishes; however, they showed a remarkable similarity in the way they oriented their tubes. Seven pointed the aperture of the primary

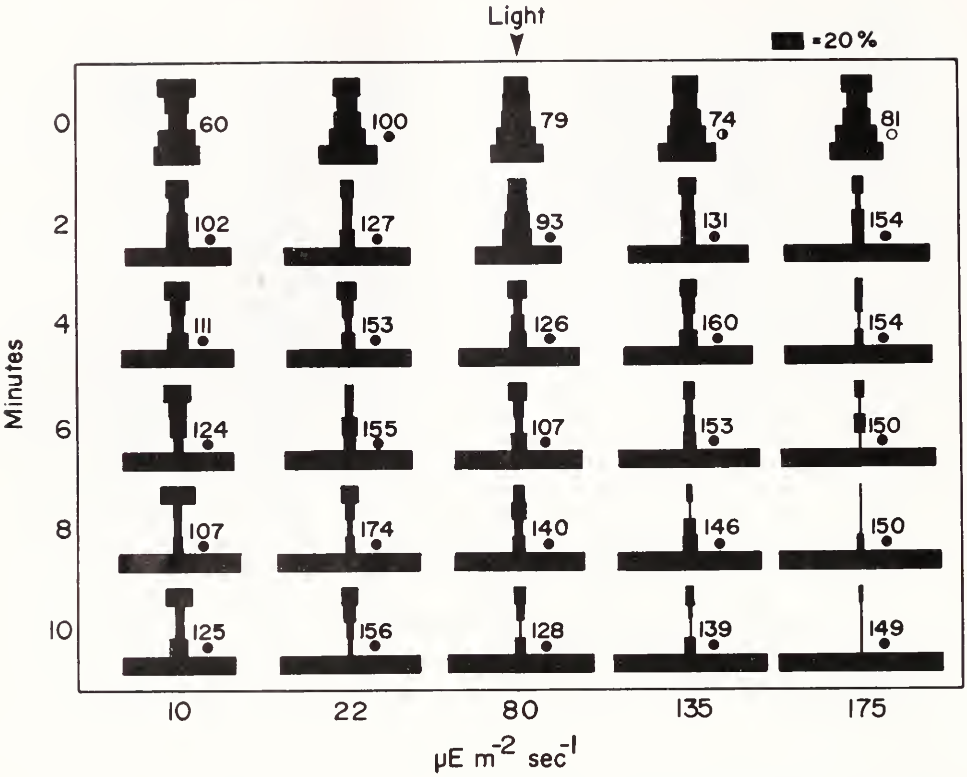


FIGURE 7. Percentage distributions of 27 day old *S. vermicularis* metatrochophores exposed to different light intensities and following different periods of light adaptation. For complete explanation, see Figure 5 legend.

tube directly away from the window, one directed it toward the light, one was angled about 45 degrees away from the light, and the remaining two tubes were roughly perpendicular to the light. If tube orientation at settlement has survival value to the juvenile worms, perhaps two ocelli function more efficiently than one in determining the light direction.

DISCUSSION

Based on laboratory observations, we predict the following movements of *Serpula vermicularis* in the field. Newly hatched trochophore larvae, which lack an eye, are indifferent to light. Nevertheless, they swim directly up, and swarm at the surface, where they remain for about a day. As this upward swimming occurs in both presence and absence of light, we assume it is a negative geotaxis. Unlike the larvae of *Hydroides norvegica*, which remain indifferent to light even after the ocellus is formed (Wisely, 1958), *S. vermicularis* larvae become strongly photosensitive as soon as the eyespot appears. These trochophores probably use light as a cue for making diel vertical migrations. The migration pattern we hypothesize resembles the "twlight" type (Cushing, 1951). Larvae swim toward the surface early in the morning, become light adapted after a short time, then swim or sink into deeper water, where they remain for most of the day. At night, low light levels

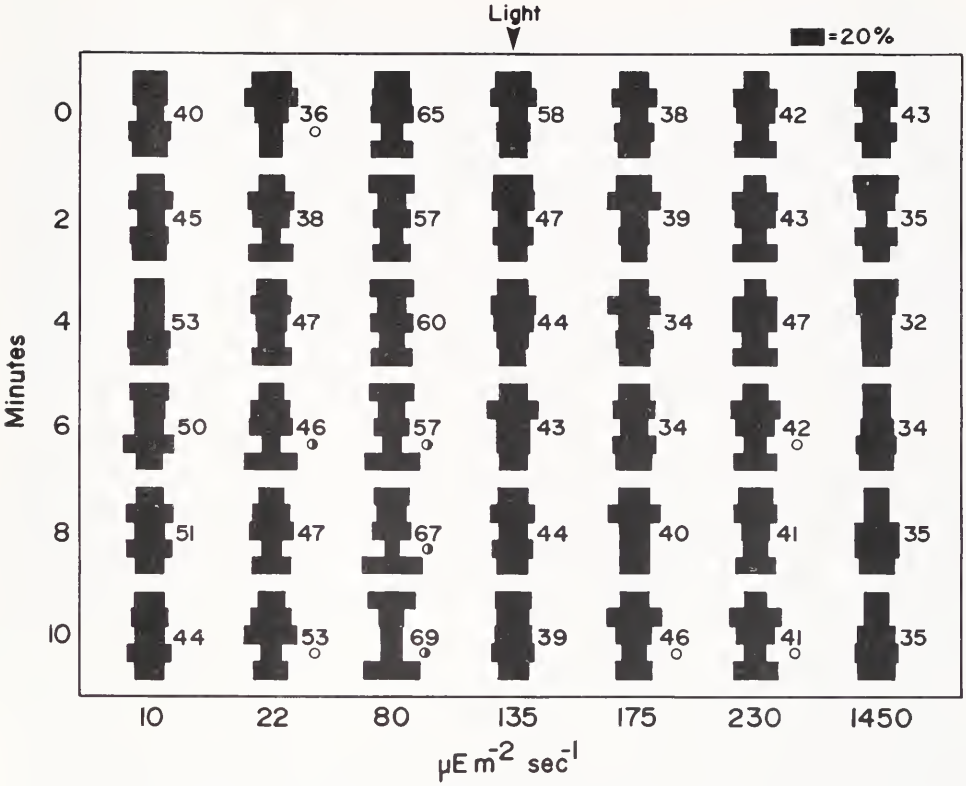


FIGURE 8. Percentage distributions of 38 day old *S. vermicularis* nectochaetes exposed to different intensities of white light and following different periods of light adaptation. For explanation, see legend in Figure 5.

may cause the larvae to disperse throughout the water column. We have no reason to suppose that there is an early evening migration to the surface, as would be predicted by Cushing's twilight migration paradigm, though evidence for this would have to come from much longer observations of light-adapted animals than we made in this study.

Like most invertebrate larvae studied to date (Forward, 1976), *S. vermicularis* larvae are sensitive to light between 500 and 600 nm, corresponding to the wavelengths which penetrate to the greatest depths in coastal waters. However, they are also capable of detecting and responding to wavelengths as short as 350 nm, which are in the ultraviolet range of the spectrum.

With the development of a second eye in the metatrochophore stage, diel migrations probably cease. The strong and constant negative phototaxis exhibited by these larvae may help them locate the bottom, as in other late stage invertebrate larvae (Thorson, 1964). Although nectochaete larvae, which are benthic, retain both eyespots, they demonstrate no phototatic response. While it may seem paradoxical that two well-developed photoreceptors would not be used during this exploratory stage, Zeleny (1905) has suggested that ocelli may be present during this period so that the juvenile shadow response, in which worms withdraw into the tube in response to an abrupt decrease in light intensity, can begin shortly after the primary tube is secreted. The setigerous larvae remain indifferent to light until after selecting a suitable substratum, at which time they apparently use light direction as a cue for orienting the tube toward the dark.

Although many workers assume that invertebrate larvae are incapable of undertaking vertical migration because of their feeble swimming abilities and the overwhelming influence of discontinuity layers in the water column (*e.g.*, Banse, 1964), others have presented convincing lab and field evidence that such migrations are at least possible (Konstantinova, 1966; Mileikovsky, 1973). The strongest support to date for the migration hypothesis comes from phyllosoma larvae in which predictions on movements made in the laboratory (Ritz, 1972a) are borne out by studies of diel changes in vertical distribution carried out in the field (Ritz, 1972b; Rimmer and Phillips, 1979). Among polychaete larvae, only the spionid *Polydora ciliata* is known to make twilight migrations (Daro, 1973). The larvae of *Mesochaetopterus sagittarius* demonstrate a "reverse" migration pattern in which they are attracted to the surface during the day and disperse at night (Bhaud, 1969). This is the migration pattern which would be expected for continuously photopositive larvae. Although field data on other potentially migrating larvae are sparse (reviewed by Young and Chia, in press), laboratory studies suggest that twilight migration could be typical of barnacles (Crisp and Ritz, 1973), crabs (Forward and Costlow, 1974), and bryozoans (Lynch, 1947). In all these species, larvae are photopositive following dark adaptation, then switch the sign of the response at high light intensities or following prolonged light adaptation. In some forms, switching to photonegative is mediated by an interaction with temperature (Ott and Forward, 1976) or salinity (Latz and Forward, 1977), though in *S. vermicularis*, it would appear that factors other than phototaxis need not be invoked to predict a twilight migration pattern.

A number of "ultimate factors" have been proposed to account for the widespread phenomenon of vertical migration in zooplankton. These hypotheses have been reviewed by McLaren (1963) and include maximizing dispersal (Hardy and Gunther, 1935), minimizing predation (Hutchinson, 1967), and optimizing feeding (Harris, 1953). McLaren (1963) added to these his own hypothesis, later expanded by Enright (1977), which ascribes a metabolic advantage to feeding in warm surface waters and digesting at a lower temperature in deep water. In the San Juan Islands, strong currents mix the water during every tidal cycle, preventing the establishment of strong thermoclines and distributing food more or less evenly through the water column (Thompson and Phifer, 1937). Thus, of the above hypotheses, only Hutchinson's idea that larvae stay deep to avoid visual predators would seem to apply to the presumed migration of *S. vermicularis* larvae. Selective pressures favoring a very brief daily excursion to the surface are harder to envision. One factor which seems not to have been considered as a selective pressure on larvae in coastal waters is the high probability of encountering the bottom. Larvae reaching the substratum before becoming competent to metamorphose, whether because of currents or their own photoresponses, are likely to become trapped in the "dead spaces" between, behind, and under rocks, or in the boundary layer where water flow is negligible. Entrapment on the bottom would effectively nullify whatever advantage the larvae accrue by dispersing in the plankton for several weeks, and would also expose larvae to hazards associated with the benthos, including benthic predators, silt, attached bacteria, and possibly lower food concentrations. A short photopositive stage each day would help resuspend trapped larvae in the water column. The fact that the response is absent in the bottom-seeking metatrochophore stage is consistent with this hypothesis.

Buss (1979) has suggested that many larvae may rely on negative phototaxis to locate cryptic settlement sites where physical and biological selective pressures are less intense. While this seems to be true for many soft-bodied epifaunal invertebrates in the San Juan Islands (Young, unpublished data), the generalization cannot be extended to *S. vermicularis*, since *S. vermicularis* nectochaetes are effectively indifferent to light while seeking a settlement site. Photoresponse may be overridden during this stage by some other behavior such as contact chemoreception, which is more critical to the worm's ultimate success. *S. vermicularis* adults are often found in the open on subtidal rock walls and boulders; this is the distribution which would be expected on the basis of laboratory behavior. Intertidally, where most animals are located on the undersides of ledges or in cracks and crevices, the distribution may be caused by differential mortality or substratum choice rather than photoresponse. Preliminary data suggest that larvae orient their tubes away from the light at settlement; this behavior could conceivably function in reducing mortality caused by siltation or some other factor.

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